Post-Translational Modification on Arginine and Function of CCAAT/Enhancer Binding Protein α

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von
M. Sc. , Qingbin Liu

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Dr. h.c. Christoph Markschies

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät I
Prof. Dr. Andreas Herrmann
Gutachter: 1. Prof. Dr. Achim Leutz
2. Prof. Dr. Udo Heinemann
3. Prof. Dr. Thomas Sommer

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Summary

The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) coordinates cell cycle arrest and terminal differentiation of neutrophil granulocytes and adipocytes. Mutations in C/EBPα are frequently associated with acute myeloid leukemia. Mass spectrometric analysis revealed that citrullination occurred on multiple conserved C/EBPα arginine residues including R297 in the C/EBPα basic region. C/EBPα R297 was previously reported to be mutated in acute myeloid leukemia and we therefore focused on the modification this residue. Data presented here show that peptidylarginine deiminase 4 (PADI4) interacts with and citrullinates C/EBPα at several sites. Citrullination or mutation of R297 dramatically changed C/EBPα activities, including DNA binding and interaction with protein partners. Mutational analysis demonstrated that the positive charge of residue R297 was critical for binding to cis-regulatory sites on DNA, gene activation, adipocytic differentiation, and cell cycle arrest. Knock down of PADI4 in the myeloid precursor cell line 32D or U937 leukemia cells induced granulocyte differentiation, potentially through relieving PADI4 mediated citrullination and inactivation of C/EBPα. Taken together, the data suggest that PADI4 converts the positive C/EBPα R297 side chain to the non-charged citrulline side chain which destabilizes the association with DNA and affects C/EBPα - E2F interaction that determines the balance between proliferation and differentiation.

Keywords:

C/EBPα, E2F, Differentiation, PADI4, citrullination

Zusammenfassung

Der Transkriptionsfaktor CCAAT/enhancer-binding protein α (C/EBPα) kontrolliert Zellzyklusarrest und terminale Differenzierung von neutrophilen Granulozyten und Adipozyten. Mutationen von C/EBPα treten häufig im Zusammenhang mit akuter myeloischer Leukämie auf. Massenspektrometrische Untersuchungen zeigten, dass C/EBPα an mehreren konservierten Argininen citrunilliert ist, einschließlich R297 in der C-terminalen basischen Region von C/EBPα. Mutationen von C/EBPα R297 wurden bereits beschrieben, weshalb der Schwerpunkt dieser Arbeit auf die Analyse der Modifikation dieses Aminosäurerestes gelegt wurde. Die Ergebnisse zeigen, dass die Peptidyl-Arginin-Deaminase (PADI4) mit C/EBPα interagiert und an mehreren Aminosäureresten citrunilliert. Citrunillierung oder Mutation von R297 beeinflusst die Aktivität von C/EBPα, einschließlich DNA-Bindung und Interaktion mit Partnerproteinen. Mutationsanalysen legen nahe, dass die positive Ladung des Aminosäurerestes R297 für die Bindung an cis-regulatorische DNA-Elemente, Protein Interaktionen, Genaktivierung, Fettzelldifferenzierung und Zellzyklusarrest ausschlaggebend ist. Knock-down von PADI4 in der myeloischen Vorläufer-Zelllinie 32D oder in der leukämischen U937 Zelllinie induziert Granulozyten-Differenzierung, möglicherweise durch Blockierung der PADI4-vermittelten Citrunillierung und Inaktivierung von C/EBPα. Zusammengefasst ergibt sich aus den Daten, dass PADI4 die positiv-geladene Seitenkette von C/EBPα R297 in eine ungeladene, citrunillierte Form umwandelt, die die Assoziation mit DNA destabilisiert und die C/EBPα-E2F-Interaktion beeinflusst, was wiederum das Gleichgewicht zwischen Proliferation und Differenzierung bestimmt.

Contents

1 Introduction

1.1 CCAAT/Enhancer Binding Protein

CCAAT/enhancer-binding protein α (C/EBPα) is a member of basic region leucine zipper (bZIP) transcription factors. This family contains six members: C/EBP α, β, δ, ε, γ and ζ. All C/EBPs consist of an N-terminal transactivation domain (TAD), a C-terminal basic-amino-acid-rich DNA-binding region and a leucine zipper coiled-coil dimerization domain (Johnson, 2005; Landschulz et al, 1988; Nerlov & Ziff, 1995; Vinson et al, 1989). C/EBPε and ζ contain 2 and 4 exons respectively while others are intronless (Ramji & Foka, 2002). C/EBPα expression is restricted to liver, hematopoietic system, lung and adipose tissue (Darlington et al, 1998; Lopez et al, 2009; Wang et al, 1995; Wu et al, 1999; Zhang et al, 1997). C/EBPβ is mainly expressed in liver, lung, adipose tissue, spleen, kidney and myeloid macrophage and granulocytes. C/EBPε is limited to granulocytic cells and C/EBPδ is mainly expressed in adipose tissue, intestine and lung (Ramji & Foka, 2002).

Since C/EBPs can dimerize (homo- or hetero-) with each other and bind to the same target consensus in promoters, it is not surprising that they share similar activities and cooperate in transcription of lineage specific genes. Additionally, independent researches suggested that C/EBPα, β and ε binds to retinoblastoma protein (pRb) and arrest E2F/DP mediated transcription (Gery et al, 2004; Iakova et al, 2003; Porse et al, 2001; Sebastian et al, 2005; Slomiany et al, 2000; Wethmar et al). However, it does not necessarily mean C/EBPs compensate the functional roles that are played by other members. In myeloid differentiation, C/EBPα induces early neutrophil genes including myeloperoxidase (MPO), elastase (NE), IL-6 receptor and G-CSF receptor but the expression of C/EBPα is dramatically decreased in mature neutrophils. Expression of C/EBPβ and δ were observed in immature myeloblasts and intermediate mature myelocytes, and increased to the peak level in terminal differentiated cells (Bjerregaard et al, 2003; Nerlov, 2004;

Scott et al, 1992). It seems that C/EBPα mainly initiate granulopoiesis and cease the proliferation progress but β and δ are more relevant to the maturation and inflammatory response (Ramji & Foka, 2002). The pattern of granulocyte and adipocyte expression of C/EBP is quite different. C/EBPβ and δ are induced at an early stage in response to adipogenic hormones, followed by expression of C/EBPα in terminal differentiation. During the regulatory cascade of adipogenesis, C/EBPβ and δ regulate adipocyte specific genes, such as insulin-stimulated glucose transporter (GLUT4), peroxisome proliferators activated receptor γ (PPARγ), fatty acid binding protein (422/aP2) and even C/EBPα (Christy et al, 1989; Freytag et al, 1994; Kaestner et al, 1990). The subsequent C/EBPα expression mainly accounts for hormonal sensitivity and cooperates with PPARγ to direct terminal differentiation (Darlington et al, 1998; Wang et al, 1995; Wu et al, 1999).

The diversity of C/EBPα and C/EBPβ protein expression is increased by alternative translation initiation according to specific physiological conditions. Three proteins of C/EBPα, extended isoform, full-length (p42), and truncated isoform (p30) are generated by alternative translation initiation. The isoforms display distinct functions in proliferation and differentiation control (Muller et al; Ossipow et al, 1993). In the case of C/EBPβ, its mRNA generates three isoforms: full-length (LAP*), 21 amino-acid truncation of the amino-terminus (LAP) and large amino-terminal truncation (LIP) (Descombes & Schibler, 1991; Ossipow et al, 1993). For both C/EBPα and C/EBPβ, the amino terminal parts are responsible for co-factor binding and transactivation. As a result, the truncated p30 and LIP are generally observed as dominant negative proteins for the full-length forms especially in cell cycle regulation (Pabst et al, 2001b; Wethmar et al).

1.1.1 C/EBPα functions

As a transcription factor, C/EBPα plays important roles in liver metabolism, regeneration, hematopoietic lineage specification, neutrophil-, skin- and adipocyte differentiation (Darlington et al, 1998; Lopez et al, 2009; Wang et al, 1995; Wu et al, 1999; Zhang et al, 1997). The transactivation domain (TAD) of C/EBPα mediates interaction with TATA binding protein (TBP), CBP/p300 and SWI/SNF complex, which facilitate ATP

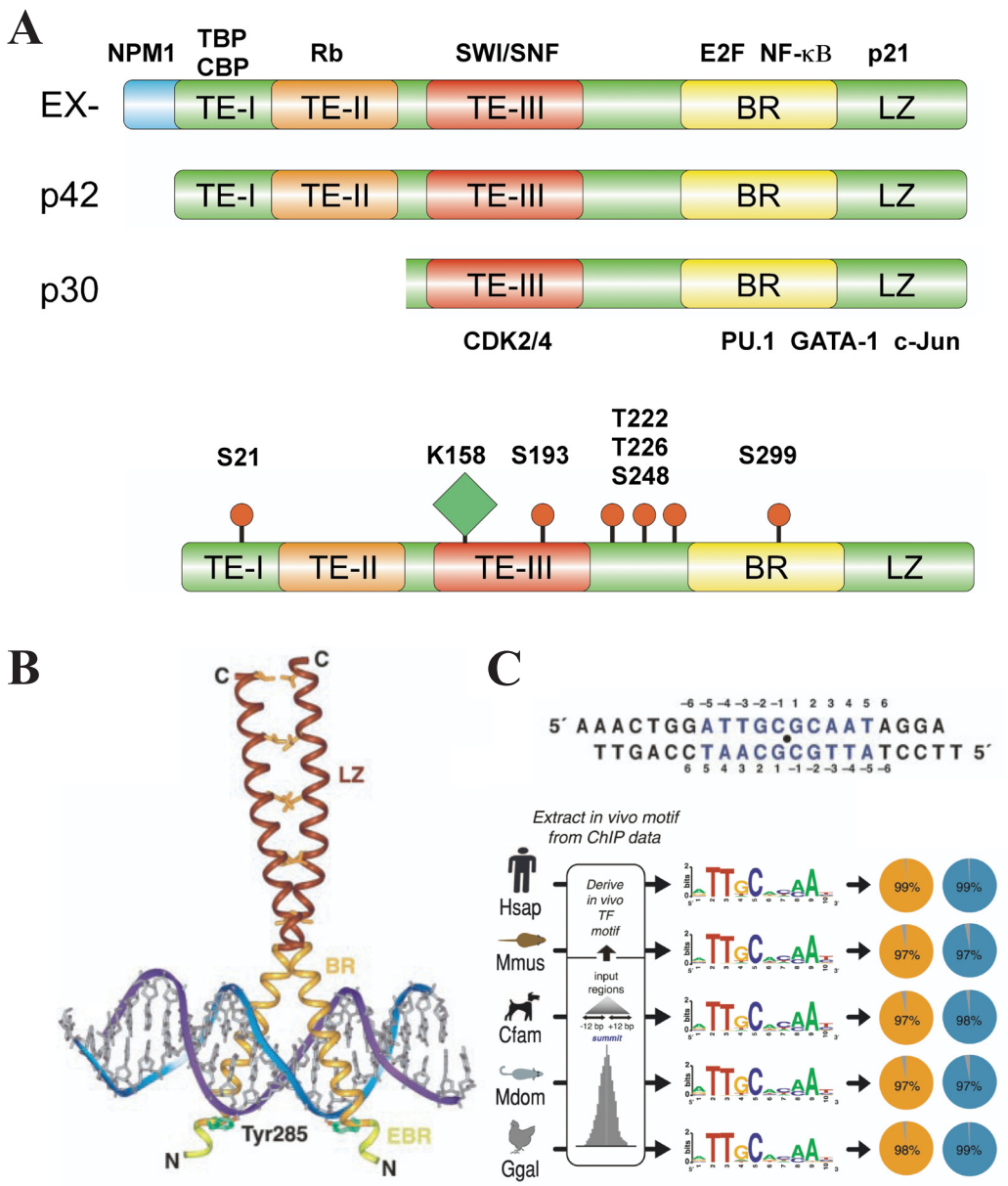

Figure 1.1 The transcriptional factor C/EBPα. (A) Schematic representation of domains contained within 3 different C/EBPα isoforms: the extended isoform (EX), full-length (p42), and truncated isoform (p30). TE= transcriptional element; TEI and II are often regarded as transactivation domain (TAD); TEIII is also named as regulatory domain (RD); BR = basic region; LZ = leucine-zipper. Interaction partners and post-translational modifications are indicated. Modified from (Johnson, 2005). (B) Crystal structure of C/EBPα bZIP dimer that is bound to a consensus DNA site, adapted from(Miller et al, 2003). (C) Up panel: Sequence of the C/EBP recognition element, adapted from (Miller et al, 2003). Down panel: Genome-wide C/EBP occupant consensus identified by chromatin immunoprecipitation with high-throughput sequencing (ChIP-seq), adapted from (Schmidt et al).

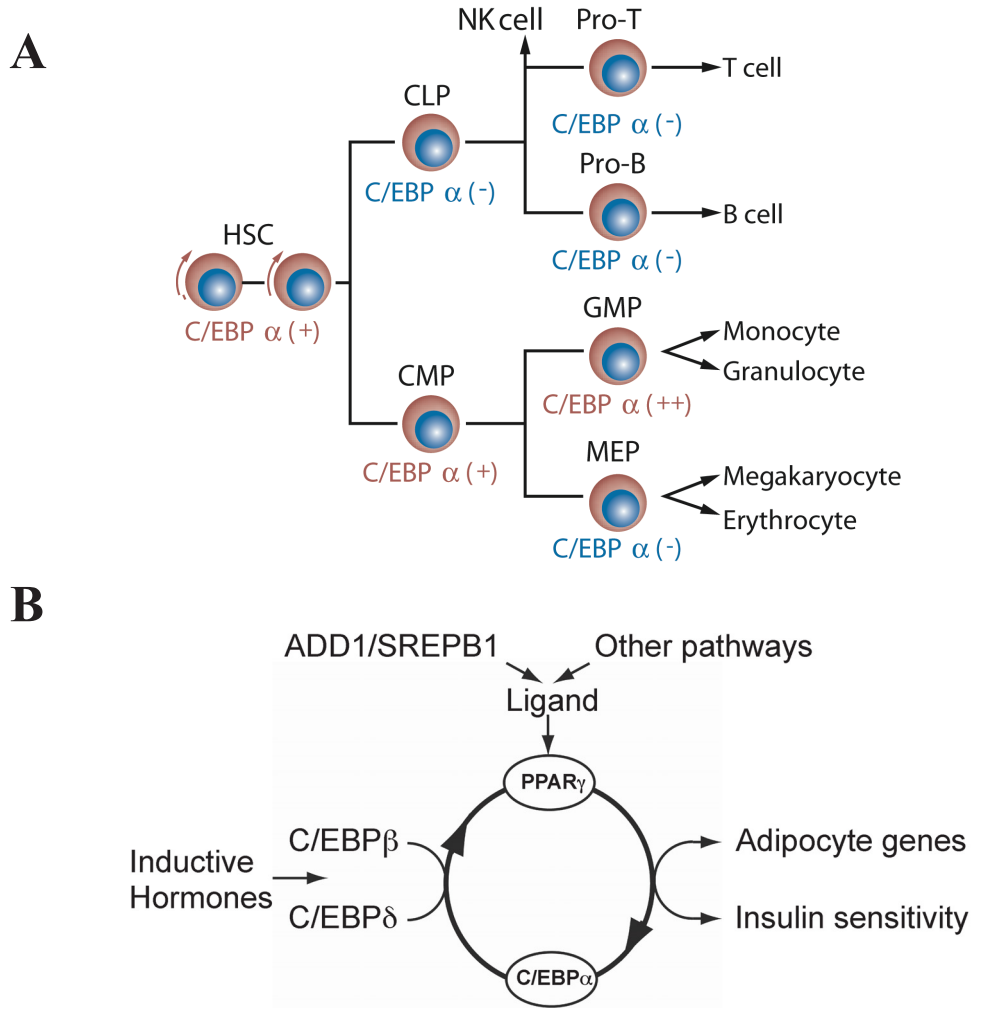

Figure 1.2 Role of C/EBPα in haematopoietic development and adipogenesis. (A) C/EBPα expression level in haematopoietic tissues. C/EBPα is expressed at low level in long-term and short-term haematopoietic stem cells. Up regulation of C/EBPα initiates the transition from common myeloid progenitor (CMP) to granulocyte/ macrophage progenitors (GMPs) and induces granulocyte differentiation. Expression of C/EBPα is undetectable in common lymphoid progenitor (CLP) and megakaryocyte/erythroid progenitors (MEPs). Adapted from (Koschmieder et al, 2009). (B) C/EBPs mediated regulatory cascade during adipogenesis. C/EBPβ and δ activates PPARγ expression during early stages of differentiation. PPARγ induces the expression of C/EBPs as well as other adipocyte genes upon ligand activation. C/EBPα and PPARγ activate each other to form a positive feedback loop. C/EBPα cooperates with PPARγ to promote adipocyte differentiation by activating gene expression and insulin sensitivity. Modified from (Wu et al, 1999).

dependent chromatin remodeling as well as basal transcription of C/EBP target genes (Nerlov & Ziff, 1995; Pedersen et al, 2001). C/EBPα cooperate or antagonize with other

transcription factors such as PU.1, GATA-1 and c-Jun in gene transactivation and lineage decisions (Friedman et al, 2003; Zhang et al, 1996). C/EBPα knock-out mice display absence of granulocytes and lack of adipocytes, which suggest an indispensable developmental role of C/EBPα in both processes (Wang et al, 1995; Zhang et al, 1997). As a key regulator in energy metabolism, C/EBPα directly transactivates insulin-stimulated glucose transporter (GLUT4) and fatty acid binding protein (422/aP2) in adipocytes (Christy et al, 1989; Freytag et al, 1994; Kaestner et al, 1990). In addition, C/EBPα knock-out mice die from hypoglycemia, due to defective induction of glycogen synthase (GS), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) genes, which are required for the newborn to establish energy homeostasis (Park et al, 1990; Wang et al, 1995).

C/EBPα mediates cell cycle arrest and resistance to tumorigenic transformation in liver, interstitial lung tissue and several other cell types (Flodby et al, 1996; Freytag et al, 1994; Hendricks-Taylor & Darlington, 1995; Iakova et al, 2003). Different pathways mediate the anti-proliferation activity of C/EBPα during the G1/S-phase transition. The eukaryotic cell cycle consists a series of events that lead to cell replication and consists of G0 phase (resting), G1 phase, S phase (synthesis), G2 phase (interphase) and M phase (mitosis). During S phase, DNA and histones are synthesized and duplicated. Early gene 2 factor (E2F) family transcription factors, together with their dimerization partner (DP), regulates G1/S phase transition and S phase progression in cell cycle. The activation of E2F1, E2F2 or E2F3b induces a group of S-phase genes such as DNA polymerase, thymidine kinase (TK), dihydrofolate reductase (DHFR) and c-myc. Other E2F members E2F4-E2F8 are generally considered as repressors on the target genes. In the G0/G1 phase, C/EBPα directly binds to E2F1and DP1, resulting in repression on E2F target genes and block of proliferation (Slomiany et al, 2000). Further studies demonstrated that N-terminal part and some critical residues in basic region of C/EBPα are critical for inhibiting E2F activity, which will be explained in more detail in section 1.1.3. On the other hand, E2F-DP impairs C/EBPα mediated adipogenesis and granulopoiesis by inhibiting its DNA-binding activity. The model proposed that E2F-DP and C/EBPα functions as differentiation-proliferation switches by repressing each other. In addition to

direct inhibition, C/EBPα regulates microRNA miR-34a, which targets E2F3 and blocks myeloid cell proliferation (Pulikkan et al). C/EBPα also suppress cell cycle progression via cyclin dependent kinase (CDK) and retinoblastoma protein (pRB) pathway. The pocket protein family consists of three proteins: retinoblastoma protein (pRB), retinoblastoma-like protein (p107) and retinoblastoma-like protein 2 (130). In G0/ early G1, pRB tumor suppressor associates with E2F and arrest E2F-DP mediated transcription. In cell cycle, CDK2 and 4 respectively associate with cyclin D1 and cyclin E to subsequently phosphorylate pRB. Subsequent release of phosphorylated pRB from E2F transcription factor leads to de-repression of S-phase genes. In this process, C/EBPα can repress CDK2/4 kinase activity through direct binding and disrupting cyclin-CDK association (Wang et al, 2001). Furthermore, C/EBPα suppress CDK by stabilizing CDK inhibitor p21 and promoting CDK proteasomal degradation (Timchenko et al, 1996; Wang et al, 2002).

In cells lacking endogenous SWI/SNF core subunit Brm, C/EBPα fails to inhibit proliferation. It seems that SWI/SNF mediated chromatin remodeling is required not only for C/EBPα target gene transcription, but also C/EBPα dependent cell cycle arrest (Muller et al, 2004). Nevertheless, it does not necessarily mean the anti-proliferation and differentiation activities are always coupled. For example, the E7 oncoprotein of the "high-risk" human papilloma virus 16 (HPV16) compromises C/EBPα-induced cell cycle arrest but does not affect C/EBPα induced adipocytic differentiation (Muller et al, 1999).

1.1.2 Singling dependent post-translational modifications on C/EBPα

The function of C/EBPα is dynamically regulated by a variety of signals and post-translational modifications. C/EBPα is SUMOylated on lysine 158 by UBC9 with attenuated association to SWI/SNF complex (Khanna-Gupta, 2008; Subramanian et al, 2003). C/EBPα is phosphorylated by ERK signal on Serine 21 with inhibition of granupoiesis while phosphorylation by Ras on Serine 248 promotes granulocyte differentiation (Behre et al, 2002; Ross et al, 2004). Phosphorylation on Serine 193 is required for C/EBP α to bind to CDK2/4 and also Brm. Signaling through phosphatidylinositol 3-kinase (PI3K)-Akt pathway induces dephosphorylation on this site

and thus disengages growth arrest (Wang et al, 2004a). In the basic region, Serine 299 was shown to be phosphorylated by protein kinase C (PKC) via tumor necrosis factor alpha (TNF-α) with attenuated site selective DNA binding (Diehl et al, 1995; Mahoney et al, 1992). Subsequently, researches suggested that this signal dependent phosphorylation modulate C/EBPα subcellular localization. In particular, phosphorylation of serine 299 in extended-isoform C/EBPα stimulates nucleolus retention and rDNA transcription (Muller et al; Yin et al, 1996).

1.1.3 Disruption of C/EBPα and leukemogenesis

Deregulation of cell cycle may lead to tumor formation. In specific subgroups of leukemia patients, C/EBPα controlled anti-proliferation activities are disrupted by different pathways on multiple levels. Understanding the mechanisms underlying C/EBPα functions may contribute to shed light on leukemogenesis and provide therapeutic strategies in acute myeloid leukemia.

At the transcriptional level, hypermethylation of C/EBPα distal promoter elements (-1600 to-600 from ATG) with silenced CEBPA transcription occurs in 30% of AML patients (Lin et al, 2010). Those methylated AML cases share similar phenotypic features with bi-allelic CEBPA mutations but no association with CEBPA mutations were observed (Szankasi et al, 2010). In addition, certain cases of acute myeloid leukemia (AML) French-American-British (FAB) M2 subtype is characterized by t(8;21)(q22;q22) chromosomal translocation. The translocation gives rise to fusion protein AML1-ETO, which suppresses AML1 and C/EBPα dependent transcription as well as C/EBPα mRNA level through disrupting positive autoregulation (Pabst et al, 2001b). C/EBPα mRNA is down regulated in posttranscriptional manner by miRNA-124a (Hackanson et al, 2008).

Inhibitory factors on C/EBPα translation were also reported. The disruption of CEBPA gene by t(14;19) chromosomal translocation with immunoglobin H locus leads to aberrant C/EBPα protein expression in lymphoblastic acute leukemia. In chronic myeloid leukemia (CML) that are caused by t(9;22)(q34;q11) chromosomal translocation, p210 BCR-ABL protein is generated. The BCR-ABL oncoprotein initiates hyperproliferation

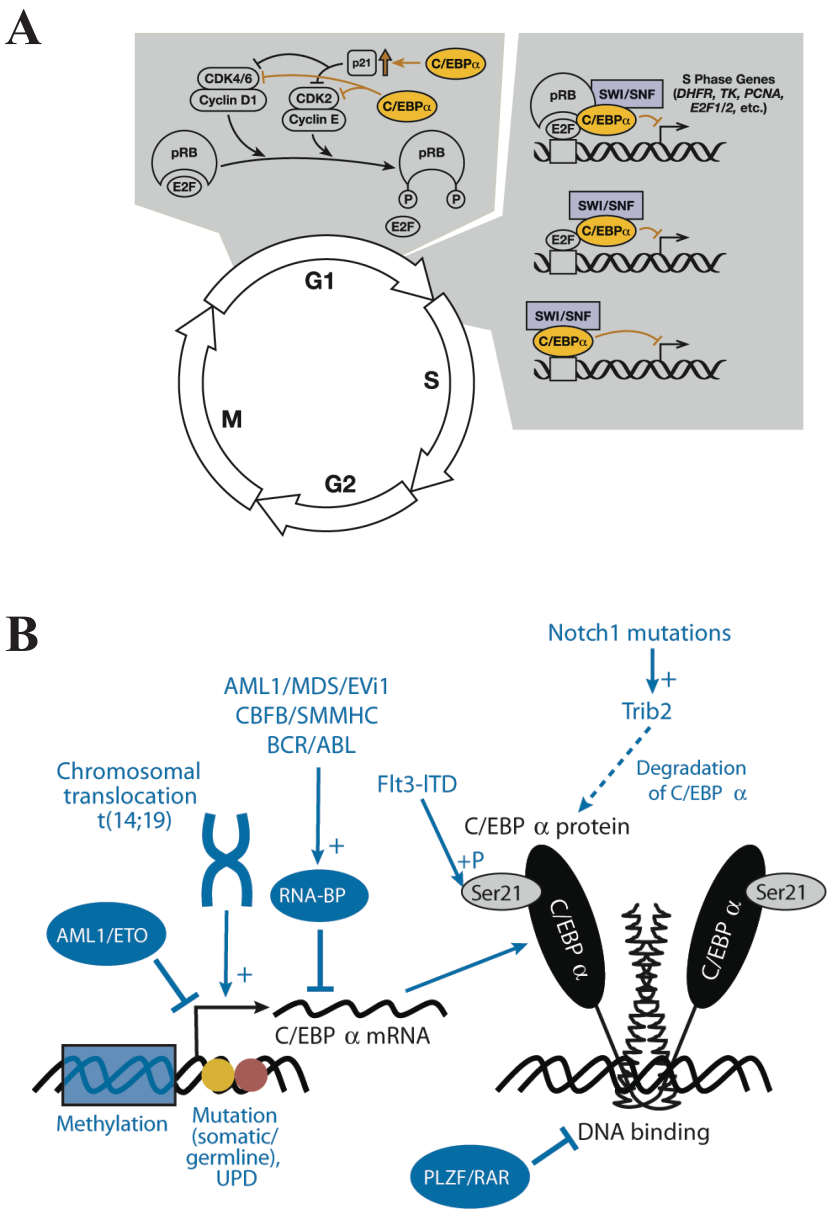

Figure 1.3 C/EBPα mediated proliferation control and disruption of in C/EBPα in leukeamogenesis. (A) C/EBPα arrest cell cycle at G1-S phage boundary via multiple pathways. Unphosphorylated Rb binds to E2F and inhibits E2F mediated transcription. C/EBPα inhibits CDK2/4 and stabilizes CDK inhibitor p21 to prevent phosphorylation on Rb protein. Moreover, C/EBPα directly interacts with E2F/DP and suppress E2F mediated S-phage gene activation. Chromatin remodeling or DNA binding competition on E2F target genes is involved. Adapted from (Johnson, 2005). (B) C/EBPα activities are disrupted by onco-proteins on transcriptional, translational and posttranslational levels in leukemia. Mutations on C/EBPα gene are also frequently observed in leukemia. Details see text 1.1.3. Adapted from (Koschmieder et al, 2009).

of white blood cells through constitutively activated tyrosine kinase activity (Puil et al, 1994). It leads to genomic instability by inhibiting DNA repair and processes the leukemia to blast crisis (BC) stage (Burke & Carroll). Moreover, the RNA binding protein hnRNP E2, which is induced by BCR-ABL via MAPK pathway, can bind to upstream open reading frame of C/EBPα mRNA and inhibits the protein translation (Perrotti et al, 2002).

C/EBPα activity can be disrupted at the posttranslational level. Tribbles homolog 2 (Trib2), whose expression is increased in Notch1 mutations in AML, promotes C/EBPα protein degradation and perturbs myeloid development in mouse. In AML patient samples, elevated Trib2 expression is observed and associates with a high frequency of C/EBPα mutations (Keeshan et al, 2006). Constitutively activating FLT3 kinase mutations are detected in around 30% of AML patients. The Flt3 internal tandem duplication (Flt3-ITD) phosphorylates C/EBPα on serine 21 by activating ERK1/2 pathway (Radomska et al, 2006). Although C/EBPα mutants mimicking this posttranslational modification (S21D, S21E) retain certain transactivation, then can not activate chromatin embedded genes. Thus granulocytic differentiation is blocked and shifted to monocytic phenotype (Ross et al, 2004).

Many studies revealed mutations in the C/EBPα coding sequence in approximately 9% of AML patients. The mutations are predominantly found in the M1 and M2 FAB morphological subtypes (around 20%) (Gombart et al, 2002; Snaddon et al, 2003). These mutations usually accompany with FLT3-ITD and NPM1 mutations but do not associate with CEBPA promoter hypermethylation or t(8;21) translocation (Pabst et al, 2001b; Szankasi et al; Wouters et al, 2009) . Most patients with CEBPA mutations had biallelic mutations involving the N-terminal transactivation domain (TAD) on one allele and C-terminal bZIP region on the other. Patients with such bialletic CEBPA mutations have low amount of leukocytes and show a distinct immunophenotype with significantly increased expression of CD7, CD15, CD34, and HLA-DR (Barjesteh van Waalwijk van Doorn-Khosrovani et al, 2003; Lin et al, 2005; Wouters et al, 2009). Further studies pointed out that TAD mutations disrupt translation of full length 42 kDa C/EBPa and

initiate translation at an alternative internal ATG codon to express 30 kDa C/EBPap30 isoform. The truncated p30 isoform lacking the amino terminal transactivation part shows reduced binding to C/EBP target promoters and may exert dominant inhibitory effects by heterodimerization with differentiation inducing, transactivation competent C/EBPs (Kirstetter et al, 2008; Pabst et al, 2001b). On the other hand, the C-terminal mutations are usually in-frame insertion/ deletion/ substitution mutations located at basic region, leucine zipper and the conjunction in between (Benthaus et al, 2008; Gombart et al, 2002). The mutations on critical amino acids may disturb DNA binding or altered dimerization/ binding with its partner proteins (Asou et al, 2003; Porse et al, 2001). Mouse mutants that harbor the amino terminal p30, or biallelic K313KK mutant in conjunction with p30, develop proliferative myeloid disorders that faithfully reflect development of human AML (Bereshchenko et al, 2009; Kirstetter et al, 2008). In summary, abrogation of the tumor suppressive function of C/EBPα is a salient feature of acute myeloid leukemia (AML) subtypes and encompasses dysregulation of the p30 isoform and mutations in the bZIP domain (Dedhia et al; Gombart et al, 2002; Koschmieder et al, 2009; Nerlov, 2004; Pabst et al, 2001b; Perrotti et al, 2002; Renneville et al, 2009; Snaddon et al, 2003).

Accumulating studies illustrated the molecular mechanisms of C/EBPα mutations in leukemia. One direct model suggested that p30 fails to repress E2F and losses the control of proliferation. Additionally, C/EBPα p30 induces a group of proteins that inhibit full-length C/EBPα activities. By using a proteomic screening of K562 cells expressing inducible C/EBPα, several proteins are induced by p30 and lead to block of granulocyte differentiation. Elevated expression of peptidyl-prolyl cis/trans isomerase (PIN1) stabilizes c-Jun, which suppresses granulocyte differentiation mediated by C/EBPα (Pulikkan et al). The induction of Ubc9 causes small ubiquitin-related modifier conjugation (SUMOylation) of full-length C/EBPα and negatively regulates C/EBPα transactivation, possibly via inhibiting SWI/SNF association (Geletu et al, 2007; Khanna-Gupta, 2008). In the p30 mouse model, a group of genes are up regulated while some other are suppressed in granulocyte-macrophage progenitor (GMP) comparing to that

from wild type (Kirstetter et al, 2008). These reports suggested an expression signature of committed myeloid leukemia cells initiated by C/EBPα p30.

C/EBPα C-terminal mutants are frequently identified in AML patients. A lot of efforts were conducted to explain the association of C/EBPα C-terminal mutants and AML phenotype. At the experimental level, certain basic region mutations serve as a good model to study myeloproliferative disorder for C-terminal AML C/EBPα mutants. It was proposed that several residues in C/EBPα basic region face away from DNA and mediate E2F binding. The basic region mutations (BRMs): BRM2 (I294A, R297A) and BRM5 (Y285A) fail to repress E2F mediated S-phase genes transcription but retains transactivation potential (Keeshan et al, 2003; Wang et al, 2003). BRM2 knock-in mice develop AML-like myeloproliferative disorders with a block of granulocyte and defective adipogenesis (Porse et al, 2005; Porse et al, 2001). On the other hand, E2F-DP complex suppress C/EBPα transcriptional activity by interfering with its DNA binding ability (Zaragoza et al). The leukemic C/EBPα BRM2 and BRM5 displays abnormal interaction with E2F-DP or C/EBPα target DNA consensus and both mutants are more amenable to E2F mediated disruption of differentiation gene induction (Miller et al, 2003; Zaragoza et al). However, the capacity of such C/EBPα mutants binding to DNA or E2F is still in debate (D'Alo et al, 2003; Keeshan et al, 2003; Zaragoza et al, 2010). From the crystal structure analysis, it is clear that C/EBPα Y285 makes critical contact with DNA and stabilizes DNA-protein interface. R297 interacts with C/EBPα target DNA backbone phosphate of G^1 and thus contributes to DNA binding ability (Miller et al, 2003). In agreement with structure analysis, both C/EBPα BRM5 (Y285A) and BRM2 (I294A, R297A) proteins from knock-in mice show cis-regulatory site specificity and fail to associate with E2F-C/EBP site from DHFR promoter in gel shift assay (Porse et al, 2001). This implies that the impaired DNA binding may account for the failure to repress on E2F-DP mediated transcription. In summary, these studies suggest that the balance of C/EBPα and E2F-DP complex is critical to adjust the switch between proliferation and differentiation.

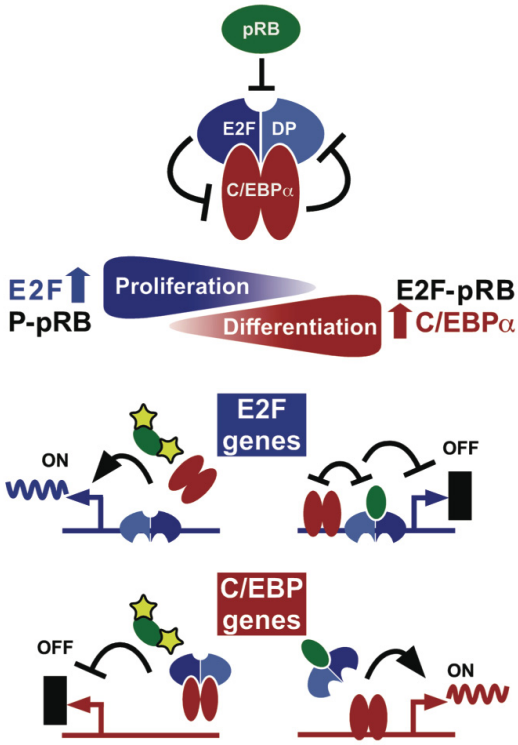

Figure 1.4: The mutual inhibition of C/EBPα-E2F/DP activities. Model to illustrate how E2F/DP and C/EBPα interplay with each other and control the proliferation/differentiation switch. E2F/DP complex is involved in induction of proliferation related S-phage genes and mediates the disassociation of C/EBPα from both E2F sites and C/EBP sites. On the other hand, C/EBPα represses the transcriptional activity on E2F-sites to arrest proliferation/ cell cycle while activates C/EBP-target genes to direct differentiation process. However, C/EBPα BRM2 and BRM5 are constitutively inhibited by E2F-DP. Thus, C/EBPα BRM2 and BRM5 fail to induce the switch towards differentiation, due to its failure to bind and repress E2F sites and to bind C/EBP sites. Adapted from (Zaragoza et al, 2010).

1.2 Post-translational modifications of Arginines

Post-translational modifications (PTM) of transcription factors and histones are consequences of extra cellular signals and play pivotal roles in functional changes protein. Divers signals mediate multiple PTMs, such as phosphorylation, ubiquitination, acetylation, SUMOylation and methylation, to regulate protein activity, localization, stability and protein partner interaction (Farley & Link, 2009). PTMs in chromatin

associated histones are best studied and summarized as " Histone Code", proposing such modifications work in system to reach the final decision on transcription and cell fate (Jenuwein & Allis, 2001). Accumulating researches has demonstrated that such regulatory mechanism occurs on both histone and non-histone proteins to modulate cellular process such as transcriptional regulation, signal transduction, RNA processing and DNA repair. Here, we focus on post-translational modifications on arginines: citrullination and methylation.

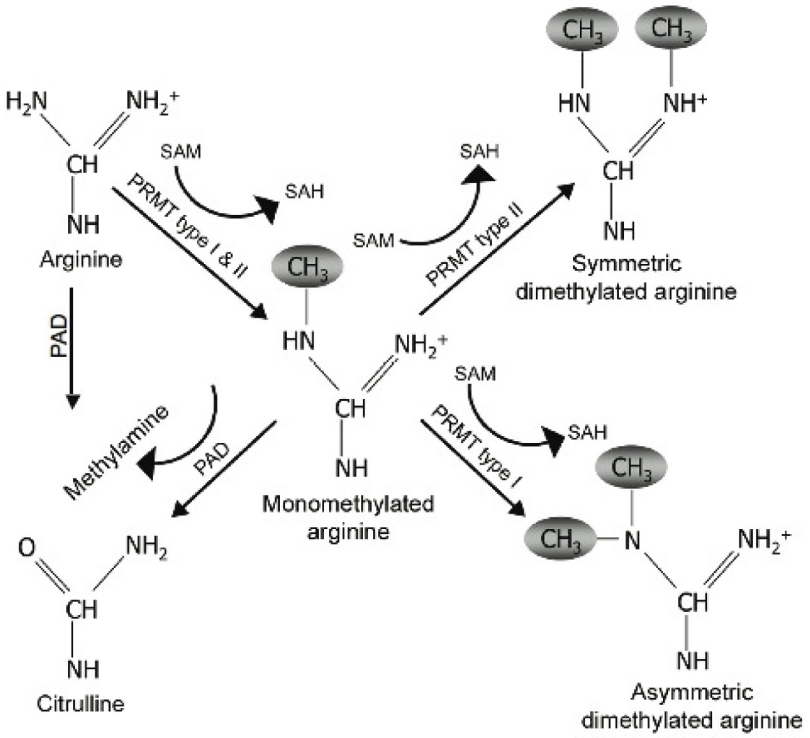

Figure 1.5 Overview of PTMs on arginine. Peptidylarginines can be monomethylated on the side chain guanidine nitrogen atom by type I and type II arginine methyltransferases (PRMTs). S-adenosyl-methionine (SAM) serves as the donor for methyl group. Type I enzymes catalyze the addition of a second methyl group to the same nitrogen atom (asymmetric dimethylation) while Type II PRMTs add a second methyl group to the opposite nitrogen atom (symmetric dimethylation). The peptidylarginine deiminase protein family (PAD) can remove an imine or methylimine group (deimination or demethylimination) from arginine or monomethyl-arginine side chain and converts the positively charged, hydrophobic arginine into neutral, hydrophilic citrulline (Cit, a nonconventional amino acid). This reaction is referred to as citrullination or deimination. Adopted from (Boisvert et al, 2005).

1.2.1 Citrullination and PADIs

Arginine is a positively charged amino acid with pKa=12.48 that is often involved in hydrogen bond formation and interaction with protein or nucleic acids. A posttranslational modification of arginines was first described with the identification of the amino acid citrulline in proteins, which was referred to as deimination or citrullination (Rogers, 1962; Rogers & Simmonds, 1958). The posttranslational citrullination/deimination in protein removes an imine group from arginine side chain and converts the positively charged arginine into neutral, hydrophilic citrulline (Cit), resulting in significant effects on protein. The loss of the positive charges may change the overall charge distribution, isoelectric point, hydrogen bond, protein tertiary structure and protein-protein interactions (Gyorgy et al, 2006; Lee et al, 2005; Vossenaar et al, 2003). This reaction is catalyzed by peptidylarginine deiminase (PAD) family and is calcium dependent (Vossenaar et al, 2003). Five members of PADIs are identified with distinct tissue distribution and specific substrates. PAD1 is mainly expressed in the spleen, thymus, epidermis and citrullinates keratin. PADI2 is broadly distributed and highly expressed in nervous system, skeletal muscle and myeloid monocyte with myelin basic protein (MBP) and vimentin as substrates. Although PADI2 is mainly located in cytoplasm, PADI2 may also catalyze citrullination of histones (Cherrington et al). PAD3 is found in hair follicles to cause modification on trichohyalin. PAD6 is expressed in eggs, ovaries and the early embryo but the function is not clear. Among the PADIs that are identified in mammals, PADI4 (also known as PADI5) is the only one that contains a nuclear localization signal (NLS) and associates with euchromatic regions of the myeloid neutrophil nucleus (Hagiwara et al, 2002; Nakashima et al, 1999). Until now, nucleophosmin/B23 (NPM), p300, histone 3 and histone 4 were identified to be PADI4 substrates. It has been reported that arginine methylation and citrullination regulate the activity of p300 and coactivator complex assembly (Hagiwara et al, 2002; Lee et al, 2005). P53/PADI4 dependent citrullination on NPM1 is involved in its cellular localization and cell growth (Tanikawa et al, 2009). Citrulination of Inhibitor of Growth 4 (ING4) by PADI4 increased susceptibility of ING4 to degradation and disrupts interaction between ING4 and p53 (Guo & Fast, 2011). Of particular interest, PADI4 targets arginine 2, 8, 17, 26 of histone 3 and arginine 3 of histone 4 as citrullination

substrates, which is considered as a process to antagonize histone methylation and repress gene activation (more details in 1.2.3).

Both PADI2 and PADI4 are expressed in haematopoietic monocytes and granulocytes with potential functions that are involved in inflammatory response. PADI2 can regulate inflammatory response by modifying multiple arginine residues into citrulline of chemokines including CXCL5, CXCL8, CXCL10, and CXCL12 (Mortier et al; Proost et al, 2008; Struyf et al, 2009). These modification on chemokines resultes in impaired interaction with receptor and affects inflammation. Citrullination on CXCL5 further reduced its capacity in regulating CD11b expression and chemotactic neutrophil extravasations. Recently, IKKγ (also known as NF-κB essential modulator, NEMO) was identified as PADI2 substrate in cytoplasm. IKKγ is a regulatory component of IκB kinase (IKK) signalsome that is responsible for NF-κB activation and nucleus translocation. PAD2 binds to and citrulliates IKKγ, which results in suppression of NF-κB activity and LPS induced inflammation (Lee et al). The nucleus localized PADI4 was first identified in HL-60 cells upon induced differentiating to monocytes and granulocytes by DMSO or vitamin D (Nakashima et al, 1999). Hypercitrullination by PADI4 was reported to induce chromatin decondensation and form neutrophil extracellular trap (NET) that is implicated in immune response (Wang et al, 2009). These reports suggest a potential role of PADI4 in cell differentiation or granulocyte function. Surprisingly, when PADI4 is over expressed in HL-60 and Jurkat T cells, apoptosis is induced (Liu et al, 2006), which was suggested to be caused by hyper-citrullination of histone. Although the role of PADI4 heamatopoiesis is unclear yet, it was proposed that *in vivo* PADI4 is expressed in specific stage and attenuates priming of myeloid cell differentiation (Balint et al, 2005).

Abnormal expression of PADI4 and hyper-citrullination were suggested to be involved in multiple diseases. Accumulating number of researches found that PADI4 is elevated in CD34+ haematopoietic stem cells (HSC) and a variety of malignant tumors including leukemia, breast cancer and lung cancer (Baka et al; Chang & Fang; Chang et al, 2009). In leukemia mouse model expressing MLL-AF9 or C/EBPα p30, PADI4 is highly

expressed in HSC as well as leukemia initiating stem cells, which suggestes that PADI4 contributes to tumor transformation and playes a role in cell self-renewal (Kirstetter et al, 2008; Krivtsov et al, 2006). Increased PADI4 expression in blood correlates with enhanced citrullinated antithrombin levels, which lead to elevated VEGF and integrin β3 (Chang & Fang, 2010; Chang et al, 2009). It was observed that PADI4 interacts with histone deacetylases (HDACs) to repress the tumor suppress p53 target genes such as p21 (Li et al, 2010). Recently, inhibitor of growth 4 (ING4), a subunit of a HBO1 histone acetyltransferase complex, was identified as a PADI4 substrate by microarray method and in vitro reaction (Guo & Fast). ING4 functions as a tumor suppressor by binding to p53 and enhancing p53 target genes. It was suggested that citrullination of ING4 nuclear localization signal (NLS) region impairs p53 dependent gene activation by disrupting p53-ING4 interaction and possibly by affecting cross talk with histone acetylation or methylation (Guo & Fast, 2011).

PADI4, together with PADI2, is also involved in autoimmune diseases. Abnormal expression of PADI4 and PADI2 generates cyclic citrullinated peptides (CCP) as autoantigen in rheumatoid arthritis (Takizawa et al, 2005; Yamada, 2005; Yamada et al, 2003). In neural system, the positively charged MBP interacts with negatively charged lipids to from myelin sheath surrounding neurons (Beniac et al, 2000). Citrullination disturbs MBP-lipid interaction and changes the ultrastructure (Pritzker et al, 2000). Hyper-citrullination of MBP or histones is implicated to be more susceptible to degradation, which may contribute to cell apoptosis and pathological process of multiple sclerosis (MS) (Mastronardi et al, 2006; Pritzker et al, 2000).

1.2.2 Methylation and PRMTs

Arginines in protein can also be methylated by protein arginine methyltransferases (PRMTs) (Gary & Clarke, 1998; Paik & Kim, 1967). Unlike citrullination, arginine methylation does not change the positive charge, but increase protein hydrophobicity. In the methylation process, PRMTs transfer a methyl group from methyl-donor S-adenosyl-L-methionine (SAM) to the guanidinium side chain nitrogen atom of an arginine residue to form monomethylated arginine (MMA). Subsequent methylation results in asymmetric

dimethylated arginine (aDMA), or symmetric dimethylated arginine (sDMA). Until now, 11 PRMTs have been identified. All PRMTs contain a catalytic domain and subdomains that are involved in substrate binding or activation. According to the methylation state, PRMTs can be classified into two different groups: Type I enzymes (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6, and PRMT8) catalyze the formation of MMA and aDMA; type II enzymes (PRMT5, PRMT7, and PRMT9) catalyze the formation of MMA and sDMA (Boisvert et al, 2005; Wolf, 2009). PRMTs have diverse substrates and the arginine methylation plays important roles in protein compelx regulation, chromatin remodeling, gene transcription, signal transduction, RNA processing, DNA repair and so on (Bedford & Clarke, 2009; Bedford & Richard, 2005; Lee & Stallcup, 2009; Pal & Sif, 2007).

PRMT4 is also referred to as coactivator-associated arginine methyltransferase (CARM1) since it was identified to associate with p160 coactivator GRIP1 (Chen et al, 1999). CARM1 specifically catalyze histone 3 at position H3R2, R17 and R26 (Schurter et al, 2001). Additionally, PRMT4/CARM1 can also methylate CREB (cyclic adenosine monophosphate response element-binding protein) binding protein (CBP) and p300 coactivators to modulate transcription activation/repression switch in signal dependent manner (Xu et al, 2001). Histone 3 lysine 18 and lysine 23 acetylation allows for efficient H3R17 methylation and links to gene activation (Bauer et al, 2002; Daujat et al, 2002). PRMT4/CARM1 mediated methylation on histone 3 often cooperates with PRMT1 medated histone 4 methylation to exert their coactivator function (Kleinschmidt et al, 2008; Miao et al, 2006). PRMT4/CARM1 contains a conserved protein arginine methyltransferase (PRMT) catalytic core domain and C-terminal autonomous activation domain (Teyssier et al, 2002; Yue et al, 2007). PRMT4/CARM1 activity is inhibited by phosphorylations. Phosphorylation on S228 impairs PRMT4/CARM1 homo-dimmerization, which is required for enzymatic activity (Higashimoto et al, 2007). Another phosphorylation site is on a conserved Serine 217 in all type I PRMTs. Phosphorylation on S217 abolishes AdoMet binding and arginine methyltransferase activity. S217 phosphoryaltion is also involved in PRMT4/CARM1 translocation from nucleus to cytoplasm in G2/M transition (Feng et al, 2009).

Embryos with targeted disruption of PRMT4/CARM1 are smaller in size and die perinatally due to defect in proper lung inflation (Yadav et al, 2003). It was also observed that PRMT4/CARM1-deficient MEFs fail in activation of estrogen-responsive genes and show impaired H3 methylation (Yadav et al, 2003). PRMT4/CARM1-deficient embryos displayed aberrant thymocyte development which is explained by the hypomethyaltion of thymocyte cyclic AMP-regulated phosphoprotein (TARPP) (Kim et al, 2004). Moreover, PRMT4/CARM1-knockout embryos have decreased amount of brown fat, which suggest PRMT4/CARM1 play role in adipocyte maturation by regulating various genes that are involved in lipid metabolism (Yadav et al, 2008).

PRMT4/CARM1 is regarded as a coactivator for many transcriptional factors. For example, as a PPARγ coactivator, CARM1 promotes preadipocytes to differentiate towards adipocytes (Yadav et al, 2008). By direct binding to p65, CARM1 can regulate NF-κB target gene transcription. In CARM1-deficient MEFs, NF-κB mediated expression of proinflammatory genes such as G-CSF, MIP-2, MCP-1, ICAM1 and IP-10 were abrogated (Covic et al, 2005). The recruitment of PRMT4/CARM1 to NF-κB target promoter correlates with H3R17 methylation, H3K9 and H3K14 acetylation and decreased H3 citrullination (Miao et al, 2006). The PRMT4/CARM1 mediated specific gene promoter activation generally involves its methyltransferase activity in cooperation with PRMT1 and acetylation coactivation by CBP/p300 or p160 family. However, it was pointed out the C-terminus of PRMT4/CARM1 is an autonomous activation domain independent of enzymatic activity (Teyssier et al, 2002; Yue et al, 2007). Introducing WT CARM1 or catalytically inactive form to CARM1-deficient MEFs revealed that catalytic activity is dispensable for certain genes activation. These data imply that CARM1 contributes to in protein complex stabilization and works as a scaffolding protein at a subgroup of p65-dependent gene promoters (Jayne et al, 2009).

PRMT4/CARM1 mediated protein methylation does not always result in functional activation. CARM1, together with ACTR and CBP/p300, activates nuclear receptor target genes by modifying chromatin H3 (Chen et al, 1999). However, CBP/p300 also serves as

non-chromatin substrates of CARM1. CARM1 mediated methylation in CBP/p300 KIX domain blocks the interaction between the kinase inducible domain (KID) of CREB, thus impairs CREB-dependent transcription(Xu et al, 2001). These studies suggest the regulatory role played by CARM1 to switch nuclear hormone pathway and cyclic adenosine monophosphate signaling pathway (Chen et al, 1999; Xu et al, 2001). Recently, the CCAAT enhancer binding protein C/EBPβ was identified as a methylation substrate of PRMT4/CARM1 (Kowenz-Leutz et al, 2010). Arginine methylation of C/EBPβ R3 in the activation domain inhibits the interaction with the SWI/SNF and mediator complexes, thereby negatively regulating activation of myeloid and adipogenic genes. Signaling through receptor tyrosine kinase-ras/ mitogen activated protein kinase (MAPK) phosphorylates the C/EBPβ regulatory domain and abrogates its interaction with PRMT4/CARM1, which is required for C/EBPβ activation (Kowenz-Leutz et al, 2010; Mo et al, 2004).

1.2.3 Interplay between Citrullination and Methylation

PTMs are often reversible regulated by opposing enzymes such as phosphorylation kinases and phosphatases (Hunter & Karin, 1992). Enzymes catalyzing methylation and demethylation also regulate arginine methylation. The Jumonji-domain containing enzyme JMJD6 was shown to demethylate both mono- and dimethylarginines in histone 3 and histone 4, as the first identified arginine-demethylase (Chang et al, 2007). In addition to real demethylation that converts methylarginines to unmodified form, PADIs mediated citrullination/ deimination is also regarded as a mechanism to antagonize arginine methylation. First of all, PAD4 can convert histone MMA to citrulline and prevent DMA, which is referred as demethylimination (Cuthbert et al, 2004; Wang et al, 2004b). Additionally, arginine citrullination prevents methyaltion on the target proteins to compete for substrates (Raijmakers et al, 2007). On the other hand, arginine methylation was also proposed to prevent citrullination (Hidaka et al, 2005). Nevertheless, while methylation increases bulkiness and overall hydrophobicity, citrullination changes overall charge and increase hydrophilicity.

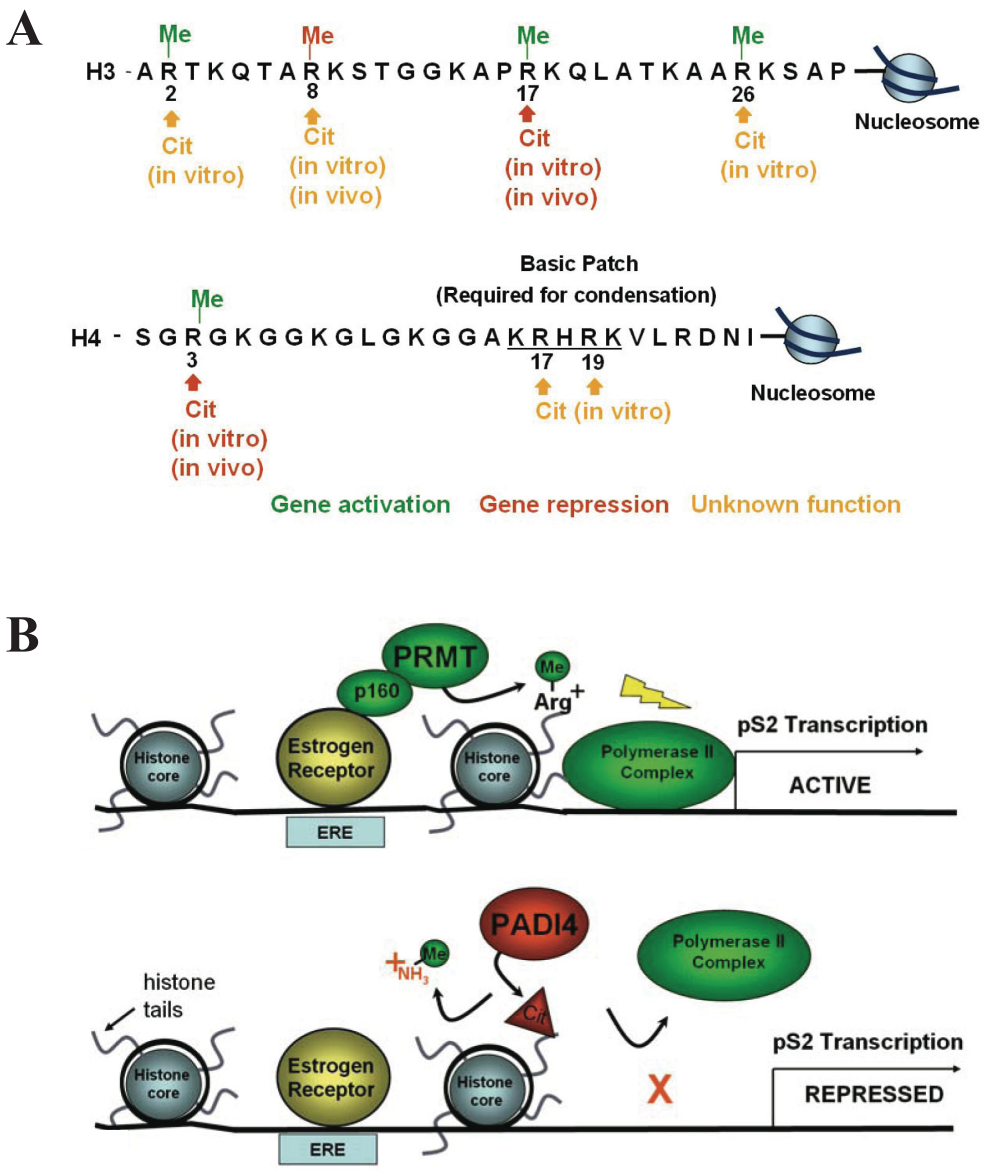

Figure 1.6 Model of antagonistic regulation of methylation and citrullination on histones. (A) Methylation sites (Me) and citrullination sites (Cit) on histone 3 and histone 4 tail. Citrullination at histone H3R17 and H4R3 are associated with gene repression. Other modifications involved in transcription or repression are also indicated. (B) Ordered recruitment of PRMT1, CARM1 and PADI4 by estrogen receptor to the pS2 gene promoter. PRMT1 methylates histone H4R3 and CARM1 methylates histone H3R17 H3R26. Such methylation leads to chromatin remodeling and coincides with the association of RNA polymerase II complex and gene transcription. Subsequent recruitment of PADI4 converts methylarginine (MeArg) to citrulline (Cit) and prevents following di-methylation. Citrullination of histone arginines decreases the ability of transcriptional machinery on chromatin template and causes repression on gene transcription. Adapted from (Wysocka et al, 2006).

The interplay of citrullination and methylation is best studied on histones. As mentioned above, histone N-terminal tails are targets for many PTMs including arginine methylation and deimination. Posttranslational modifications of histones dynamically change chromatin-associated proteins and chromatin structure (Strahl & Allis, 2000). Multiple histone arginines are methylation targets: PRMT1 catalyzes histone H4 at arginine 3 (H4R3); CARM1 targets histone H3 at arginine 17/26 (H3R17/R26); PRMT5 can modify histone H3 at arginine 8 (H3R8) and H4 ariginine 3 (H4R3); PRMT6 methylates histone H3 at arginine 2 (H3R2) and histone H4 arginine 3 (H4R3). PRMT5 and PRMT6 mediated histone methylation are thought to be linked with transcription repression, while PRMT1 and CARM1-mediated methylation correlates with gene activation (Bedford & Clarke, 2009; Wysocka et al, 2006). In addition to PRMTs, PADI4 was reported to modify histone tails on these methylation targets H3R2, H3R8, H3R17, H3R26 and H4R3. Deimination on histone H2A was also identified in HL-60 cells upon calcium-ionophore stimulation (Hagiwara et al, 2005). Model of antagonistic regulation of methylation and citrullination was proposed on chromatin. Estrogen signaling regulates the pS2 gene and directs ordered and cyclic recruitment of cofactors to the promoter (Metivier et al, 2003). The recruitment of PRMT1 and PRMT4 can methylate H3 and H4 and the modification coincides with the appearance of active RNA polymerase II complex to the promoter and the activation of transcription. Subsequent recruitment of PADI4 to the promoter would allow for the conversion of mono methyl-arginine residues to citrulline and prevent the subsequent di-methylation (Cuthbert et al, 2004). The appearance of citrulline on H3 and H4 induces conformational changes in the nucleosome and correlates with the disappearance of arginine methylation, which resulted in the disengagement of RNA polymerase II and transcription shut off (Wysocka et al, 2006). Such interplay of PRMT/PADI regulated modifications on chromatin was also observed in myeloid and NF-κB target genes (Balint et al, 2005; Miao et al, 2006).

In addition to chromatin modification and transcription, citrullinaiton/ methylation interplay is also observed on non-histones and functions in a number of different cellular pathways. PRMT4/CARM1 methylates p300 at arginine 2142 within the C-terminal glucocorticoid receptor interacting protein (GRIP1) binding domain (GBD) and inhibits

the interaction of GRIP1 to p300. This methylation is converted by PADI4 demethylimination and thereby enhanced GRIP1-p300 interaction (Lee et al, 2005). Therefore, coactivator complex assembly is switched according to the structural change of GRIPI by methylation or citrullination. Citrulliation and methylation also play role in protein synthesis. Protein arginine methyltransferase 3 (PRMT3) binds to and methylate ribosomal protein S2 (RPS2) (Swiercz et al, 2005). PAD4 citrullinates the Arg-Gly repeat region of RPS2, which is also the arginine methylation site by PRMT3 (Guo et al). Additionally, both PADI4 and PRMT3 associate with 40S ribosomal subunit fraction form cell extracts. This suggests that citrullination and methylation may antagonize each other in ribosome assembly by regulating RPS2 activities.

In this study, we show that diverse post translational modifications (PTM), including arginine citrullination and methylation, occur on transcriptional factor C/EBPα. This implies that C/EBPα might be diversely regulated by such PTMs. Subsequently, we mainly focused on the novel citrullination and tried to illustrate such regulatory mechanism and consequences on C/EBPα. Our study suggests that PADI4 interacts with the bZIP domain of C/EBPα and causes citrullination of the N-terminus and the bZIP domain. Of particular interest, citrullination targets arginine 297 is located in the bZIP region and the mutation of this residue is involved in leukemia. The data presented here showed that arginine to citrulline conversion on C/EBPα residue 297 altered the association with DNA as well as E2F-DP complex. PADI4 is expressed in 32D myeloid precursor and leukemia U937 cells and inhibition of PADI4 by shRNA promoted granulocyte differentiation. Taken together, our results suggest that PADI4 is involved in the regulation of CEBPα function and thus in myeloid proliferation and differentiation control.

2 Materials and methods

2.1 Materials

2.1.1 General chemicals

DNA Ladder (Roth)

DNA loading dye (6 x)

15% (w/v) Ficoll 400

40 mM EDTA 0.1% (w/v)

Bromophenolblue 0.1% (w/v)

Xylene cyanol FF

Acrylamide (Biorad)

30% acrylamide/Bis Solution 29:1 (3.3% C) Store at 4°C.

TEMED (Roth)

10% APS

1 g ammonium persulphate dissolve in 10 ml deionized water. Store at 4°C.

Ampicillin-stock (1000 x)

1% (w/v) Ampicillin (Sigma-Aldrich)

Solve in deionized water. Filter through 0.2µm nitrocellulose filter. Store at -20°C.

Zeocin (InvivoGen)

X-Gal (Roth)

40 mg/ml solved in N,N-dimethylformamide Store at -20°C.

IPTG (Roche)

Solve as 1 M solution in deionized water. Store at -20°C.

10 x PBS

1.4 M NaCl

0.027 M KCl

0.018 M KH_2PO_4

0.1 M Na_2HPO_4

Dissolve in deionized water, autoclave and store at RT.

Running buffer (10 x)

250 mM Tris

2 M Glycine

35 mM SDS

Dissolve in deionized water and store at RT.

SDS loading buffer (6 x)

600 mM DTT

350 mM Tris pH 6.8

10% SDS

10% glycerol

0.1 mg/ml bromophenol blue

Solve in deionized water.

Coomasie solution

1 g Coomasie Brillant Blue (FLUKA)

200 ml methanol

50 ml acetic acid

250 ml deionized water

Destain solution

200 ml methanol

50 ml acetic acid

250ml deionized water

Transfer buffer

25 mM Tris base

190 mM glycine

20% methanol

Dissolve in deionized water and add methanol. Store at 4°C.

Ponceau S

1% Acetic acid 0.5% (w/v)

Ponceau S

Dissolve in deionized water.

Tween-20 (Sigma)

PBS-Tween

Make 1:10 of 10 x PBS stock, add 0.1% (v/v) Tween.

5% non-fat milk (blocking solution)

5g non-fat milk powder (Merck) dissolved in 100ml 1 x PBS-Tween.

Roti-Block (Roth)

Dilute 1:10 in deionized water

2.1.2 Enzymes and proteins

Restriction endonucleases

All restriction endonucleases used (BamH I, Bgl II, EcoR I, EcoR V, Hind III, Not I, Xba I) were purchased from Roche.

Klenow Enzym (Roche)

PfuTurbo DNA Polymerase (Stratagene)

Lysozym (Serva)

T4 DNA-Ligase (Roche)

Alkalische Phosphatase, Shrimp (Roche)

Albumin (BSA) (Boehringer Mannheim)

Protein Ladder (Fermentas)

2.1.3 Antibodies

anti-HA antibody high affinity (3F10) (Roche)

anti-CD11b-PE (BD Bioscience)

anti-C/EBPα (14AA, Santa Cruz)

anti-FLAG M2 (Sigma)

anti-Tublin (TU-02) (Santa Cruz Biotechnology)

anti-PADI4 (K-18) (Santa Cruz Biotechnology)

anti-Modified Citrulline (17-377) (Millipore)

anti-mouse IgG HRP (GE Healthcare)

anti-rabbit IgG HRP (GE Healthcare)

anti-goat IgG HRP (Santa Cruz)

anti-rabbit IRDye 800 (Invitrogen)

anti-mouse IgG Alexa-Fluor®680 (Invitrogen)

2.1.4 Reagents and kits

Protease inhibitors cocktail (Roche)

Amersham Amplify Fluorographic Reagent (GE Healthcare)

Dynabeads Protein G (Invitrogen)

Streptavidin dynabeads (Invitrogen)

Glutathione Sepharose 4B (Amersham)

Nickel Sepharose High Performance (GE Healthcare)
HisTrap FF crude column (GE Healthcare)

High Pure RNA Isolation Kit (Roche)

Metafectene Transfection reagent (Biontex)

SuperScriptTM II Reverse Transcriptase Kit (invitrogen)

QIAGEN Plasmid Maxi Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen, Produkt-Nr. 28704)

QuikChange Site-Directed Mutagenesis Kit (Stratagene)

Enhanced chemiluminescence, ECL (Amersham)

Cell Line Nucleofector® Kit (Amaxa)

2.1.5 Cell culture solutions

Penicillin/Streptomycin (PAA)

Puromycin (InvivoGen)

100 x Trypsin-EDTA (PAA)

DMSO (Merck)

DMEM+GlutaMAXTM (Invitrogen)

MEM-AlphaMedium+GlutaMAXTM (Invitrogen)

RPMI 1640 (Invitrogen)

Fetal Bovine Serum (Gibco)

2.1.6 Equipments

Avanti Centrifuge J-25 (Beckman)

Centrifuge 5417R (Eppendorf)

BioPhotometer (Eppendorf)

Electrophoresis Power Supply (Gibco)

Agarose Electrophoresis Chamber (Roth)

Mastercycler Gradient (Eppendorf)

Thermomixer Compact (Eppendorf)

GelDoc 2000 (Biorad)

Protein Electrophoresis Chamber (Biorad)

Transfer Chamber (Biorad)

Scintillation Counter (Beckman LS 6000)

Nitrocellulose Transfer Membrane (Schleicher & Schuell)

Odyssey Scanner (Li-Cor)

Nitrocellulose Filter (Schleicher & Schuell)

Whatman Paper (Schleicher & Schuell)

X-Omat AR Film (Kodak)

Bioruptor (Diagenode)

ABI Prism 7000 (Applied Biosystems)

Accuri cytometers (BD Bioscience)

Cell Line Nucleofector device (Amaxa)

2.2 Methods

2.2.1 Working with DNA

2.2.1.1 Transformation of *E.coli*

Bacteria TOP10F' (Invitrogen) and GT116 strain (InvivoGen) was used to generate DNA plasmid. Bacteria were transformed with plasmid DNA using the heat-shock protocol. Competent bacteria were mixed and incubated with 100 ng plasmid DNA on ice for 30 min. Mixture was incubated for 45 sec at 42°C and quickly chilled on ice for 1 minute. Then 1 ml LB medium without antibiotics was added and the suspension was incubated for 1 hour at 37°C under gentle shaking. After the incubation, bacteria were gently centrifuged (3000 rpm) and plated onto LB agar plates containing the appropriated selection antibiotic. Plates were incubated overnight at 37°C. Colonies were picked and grown in LB medium with antibiotics under agitation (180 rpm, overnight, at 37°C).

BL21 (DE3) were used to produce GST fused protein or His-tagged protein. BL21 strain was transformed with DNA using electroporation with E.coli, 2mm, 2.5 KV on electroporation device (Biorad). 1 ml LB medium without antibiotics was added and the suspension was incubated for 1 hour at 37°C under gentle shaking.

2.2.1.2 DNA isolation

For testing cloned and mutated constructs, boiling miniprep method is used to isolated plasmid DNA. Briefly, the bacteria were palleted and then resuspended in STET buffer. After addition of 30 µl 1 mg/ml lysozyme, boil the mixture at 95°C for 1 min. Then spin down at 12000 rmp and remove the pellets. Add 400µl isopropanol and centrifuged at 12000rmp in 4°C for 20 min. Pellet was washed once with 80% ethanol, vacuum dried and resuspended in 50µl sterile deionized water.

STET buffer

8% Sucrose
5% Triton X-100
50mM EDTA
50mM Tris (pH 8.0)
Dissolved in sterile deionized water.

For sequencing and confirmation of insertion or mutation in plasmid (carried out by MWG, Ebersberg), small amount of highly purified plasmid DNA was isolated from a 1.5 ml bacteria overnight culture, using the Wizard®Plus SV Miniprep KIT (Promega). Large amounts of highly purified plasmid DNA were obtained from 50 ml bacteria overnight culture using the QIAGEN Plasmid MAXI Kit (Qiagen), following manufacturer's specifications.

2.2.1.3 Polymerase-Chain-Reaction (PCR) and site directed mutagenesis

The coding sequences with specific 5' and 3' restriction sites for cloning into a given construct were generated by Polymerase-Chain-Reaction. Primers for amplification were synthesized and purification by HPLC in MWG company (Ebersberg). PCR reactions were carried out using the CombiZym System (Invitek, Berlin) following manufacturer's instructions; dNTPs (Desoxi-Nucleotid-Triphosphates) were purchased from Roche. PCR products were further digested and purified as described below.

For mutating specific amino acid code in the sequence, mutagenesis was performed by PCR. The mutagenesis primers were designed at size of 33 mer with the mutation target on the center of primters. Mutagenesis was performed using the QuickChange Site-Directed Mutagensis kit (Stratagene) following manufacturer's instructions. PCR reactions were digested by DpnI enzyme overnight to disrupt template DNA. The products were then transformed into TOP10F', select clone and isolate plasmid. All mutants were confirmed by enzyme digestion followed by sequencing.

PCR primers for coloning are listed in Table 2.1. Primers for mutagenesis are listed in Table 2.2.

Cloning of	PCR Primers used for cloning
Rat C/EBPα aa1 forward	5’ cg gga tcc atg gag tcg gcc gac ttc tac 3’
Rat C/EBPα aa118 forward	5’ cg gga tcc atg tcc gcg ggg gcg cac 3’
Rat C/EBPα aa188 forward	5’ cg gga tcc ccg cac ccg cac gcg tct 3’
Rat C/EBPα aa258 forward	5’ cg gga tcc gac ctc cgc acc ggc ggc 3’
Rat C/EBPα aa278 forward	5’ cg gga tcc tcg ctc aag ggc ttg gct g 3’
Rat C/EBPα aa315 forward	5’ cg gga tcc gtg ttg gag ttg acc agt gac 3’
Rat C/EBPα aa115 reverse	5’ ccg g aat tca acc gcc ggg gcc cgc 3’
Rat C/EBPα aa193 reverse	5’ ccg g aat tcg aga cgc gtg cgg gtg cgg 3’
Rat C/EBPα aa269 reverse	5’ ccg g aat tca gcc gcc ggt gcg gag gtc 3’
Rat C/EBPα aa328 reverse	5’ ccg g aat tct gtc act ggt caa ctc caa cac c 3’
Rat C/EBPα aa359 reverse	5’ ccg ga att cgc gca gtt gcc cat ggc ctt 3’
Human PADI4 forward	5’ cg gga tcc ctg gcc cag ggg aca ttg atc cgt gtg a 3’
Human PADI4 reverse	5’ gc tct aga at tca caa gag ctc ttg ctt gcc aca ctg 3’

Table 2.1: Primers used for cloning expression constructs. A forward and a reverse primer annealing with the source plasmid were used to generate a PCR product to be inserted into target vectos. C/EBPα fragments were inserted into BamHI and EcoRI sites of the pGEX-2TK vector. Full length PADI4 was coloned into BamHI and XbaI sites of pcDNA-HA vector. aa = amino acids.

2.2.1.4 Restriction Endonuclease digestion and ligation

Restriction endonuclease digestions were performed by incubation of approximately 5 µg PCR products or plasmid with 1 µl appropriate restriction enzymes in 2 µl digestion buffer specified by the supplier at 37°C. For enzymes with star activity, the incubation time should be less than 2 hours. For cloning purposes, digested vectors were dephosphorylated for 30 min by Shrimp Alcaline Phosphatase (Roche) to avoid self-ligation. Reactions were then load on agarose gel for electrophoresis and DNA extraction. Vectors and fragments to be inserted were mixed at molar ration of 1: 3 and ligated by T4-DNA-ligase (Roche) at 14°C overnight. TOP10F’ (Invitrogen) were transformed with the ligation mixture using the Nishimura Heat-Shock protocol.

Mutation of	PCR Primers used for mutagenesis
PADI4 D350A forward	5’ gaccagtggatgcaggctgaaatggagatcggc 3’
PADI4 D350A reverse	5’ gccgatctccatttcagcctgcatccactggtc 3’
PADI4 D473A forward	5’ tccgtgggccacgtggccgagttcctgagcttt 3’
PADI4 D473A reverse	5’ aaagctcaggaactcggccacgtggcccacgga 3’
C/EBPα I294A forward	5’ cgg gaa cgc aac aac gcc gcg gtg cgc aag agc 3’
C/EBPα I294A reverse	5’ gct ctt gcg cac cgc ggc gtt gtt gcg ttc ccg
C/EBPα R297A forward	5’ aac aac atc gcg gtg gcc aag agc cga gat aaa 3’
C/EBPα R297A reverse	5’ ttt atc tcg gct ctt ggc cac cgc gat gtt gtt 3’
C/EBPα R297Q forward	5’ aac aac atc gcg gtg cag aag agc cga gat aaa 3’
C/EBPα R297Q reverse	5’ ttt atc tcg gct ctt ctg cac cgc gat gtt gtt 3’
C/EBPα R297K forward	5’ aac aac atc gcg gtg aag aag agc cga gat aaa 3’
C/EBPα R297K reverse	5’ ttt atc tcg gct ctt ctt cac cgc gat gtt gtt 3’

Table 2.2: Primers used for mutagenesis

2.2.1.5 Agarose Gel Electrophoresis and DNA Extraction

Agarose gel was prepared by boilding 1-2%(w/v) agarose in 1xTAE buffer. Once solution was cooled down to approximately 50°C, ethidium bromide (0.5µg/ml) was added and poured into casted gel chambers. Gels were run in 1xTAE, at 100V. DNA was loaded by addition of loading buffer and visualized under UV-light. For cloning purposes, DNA fragments of interest were excised with a scalpel and extracted using the QIAquick Gel Extraction Kit (Qiagen) following manufacturer’s specifications.

2.2.1.6 Plasmids

The pBabePuro based retroviral C/EBPα basic region point mutants (BRM2: I294A and R297A) were obtained from Dr. Claus Nerlov (Porse et al, 2001) and cloned into pcDNA3 plasmid as described (Zaragoza et al, 2010). WT C/EBPα fused to a carboxy-terminal FLAG was cloned into pcDNA3 vector. Individual C/EBPα I294A, R297A, R297Q and R297K mutations were introduced to by site directed mutagenesis and confirmed by sequencing. The C/EBP responsive -82 cMGF-luciferase reporter has been

described previously (Sterneck et al, 1992). HA-tagged DP1, HA-tagged E2F1 and pE2Fx6-TATA-Luciferase reporter were described previously. The coding regions of C/EBPα fragments were introduced into pGEX-2TK BamHI-EcoRI site to generate glutathione S-transferase (GST) fusion proteins. Full-length cDNA of human PADI4 (NM_012387) was purchased from ImaGenes Bioscience Company and cloned into pcDNA3-HA vector with EcoRI and XbaI sites. ShRNA oligonucleotide against PADI4 was designed by using the InvivoGen's siRNA Wizard program and inserted into psiRNA-7SKGFPzeo vector (details see 2.2.3.12 small interference RNA).

2.2.1.7 Electrophoretic Mobility Shift Assay (EMSA)

The DNA binding activity of C/EBPα and mutants were investigated by using double-stranded oligonucleotides harboring C/EBP binding sites from cMGF promoter. Oligonucleotides were synthesized and annealed to be double strand: 5'- TCGACACA ATGAGGCAAT -3'; 5'- TCGATTGCCTCATTGTG-3'. The double-strand DNA was filled up with ^{32}P dCTP by Klenow enzym to get labeled. Nuclear proteins were incubated with a 32P-labeled oligonucleotide in EMSA binding buffer for 10 min. Specific binding of C/EBPα can be supershifted by adding 1µl anti-C/EBPα antibody and incubating for additional 10 min. For competition assay, nuclear protein was pre-incubated with 50 or 100-fold excess of unlabeled oligonucleotide 10 min prior to addition of labeled probe. Adding such cold unlabeled oligonucleotide can inhibit specific binding. In the off-rate EMSA experiment, firstly incubate nuclear protein with labeled probe, then add 100-fold excess of unlabeled oligonucleotides to the reactions at specific time intervals. All of the reactions were subjected to electrophoresis on 4% non-SDS polyacrylamide gels and visualized by autoradiography.

EMSA binding buffer (4 x)

8 mM Spermidin

12 mM HEPES (pH 7.9)

12.5 mM $MgCl_2$

9.4 mM EDTA

30 µg/ml BSA

2 mM DTT

15% Glycerol

Dissolve in deionized water, store at -20°C.

EMSA reaction buffer

3 µl 4 x EMSA binding buffer

1 µl Poly(dI-dC) (2 µg/ µl)

0.5 µl KCl (1M)

H_2O up to 12 µl

EMSA loading buffer (6 x)

8% (w/v) Ficoll

500 µl TE

10 mM EDTA

0.1% (w/v) Xylene Cyanol

0.1% (w/v) Bromophenol Blue

Dissolve in deionized water, store at -20°C.

Non-SDS polyacrylamide gel (4%)

6.64 ml 30% acrylamide

5ml 10 x TBE

38.36 ml H_2O

315 µl APS 50 µl TEMED

Running in 0.5 x TBE buffer

2.2.2 Working with protein

2.2.2.1 Identification of posttranslational modifications (PTMs) in C/EBPα

The gene encoding the N-terminal His8-tagged C/EBPα (Rat) protein was overexpressed in HEK-293 cells. Whole cell lysates were prepared in lysis buffer containing guanidine hydrochloride (GuHCl). The purification procedure comprises an affinity chromatography on a 5 ml HisTrap FF crude column (GE Healthcare) charged with Ni^{2+},

and elution with buffer containing imidazol gradient. Protein samples containing C/EBPα were further purified by reverse phage chromatography with 300SB-C3 analytical column (4.6x250, Agilent Technologies). Post translational modifications (PTMs) in C/EBPα were identified by mass spectrometry in collaboration with Mass Spectrometry Core Unit, Max Delbrueck Center for Molecular Medicine.

GuHCl lysis buffer:
100 mM NaH_2PO_4
10 mM Tris
20 mM Imidazol
6 M GuHCl
1mM TCEP
Protease inhibitor
Adjust pH to 8.0 using NaOH.

Elution buffer:
100 mM NaH_2PO_4
10 mM Tris
250 mM Imidazol
6 M GuHCl
1mM TCEP
Protease inhibitor
Adjust pH to 8.0 using NaOH.

2.2.2.2 Isothermal titration calorimetry (ITC)

DNA encoding the bZIP domain of C/EBPα (both wild-type and variants), comprising the basic region and the leucine zipper (aa 281-340), was subcloned into the pQLinkH vector (Scheich et al, 2007). The gene encoding the N-terminal His7-tagged protein was overexpressed at 20 °C in E. coli Rosetta (DE3). The purification procedure comprises an affinity chromatography on a 5 ml HisTrap FF crude column (GE Healthcare), charged with Ni^{2+}, and a size-exclusion chromatography on a Superdex 75 prep grade column (26

x 60, GE Healthcare). The His7-tag was cleaved with tobacco etch virus protease prior gel filtration. ITC measurements (Wiseman et al, 1989) were performed in 20 mM HEPES pH 7.5 and 0.15 M KCl at 25°C using a VP-ITC microcalorimeter (MicroCal, LLC, Northhampton, USA). In an experiment 5 or 10 µL DNA solution (200 or 250 µM) was injected into the sample cell containing 20 µM of protein solution (monomeric wild-type and variants of alpha C/EBP bZIP domain). The DNA sequences 17/18mer CEBP-site with overhangs: forward 5' TCGACACAATGAGGCAAT 3', reverse 5' TCGATTGCCTCATTGTG 3'. A total of 25 or 50 injections were performed with a spacing of 240 s and a reference power of 18 µcal/s. Binding isotherms were plotted and analyzed using the Origin 7.0 Software (MicroCal, LLC) (N, stoichiometry; K, association constant; H, enthalpy; S, entropy). The calculated Kd-values correspond to 1/K. ITC measurements were performed in collaboration with Protein Sample Production Facility, Max Delbrueck Center for Molecular Medicine.

2.2.2.3 Purification of GST-fusion proteins

BL21 (DE3) bacteria were electroporated with the pGEX-2TK prokaryotic expression constructs encoding Glutathione S-transferase (GST) or GST fused C/EBPα fragments. Bacteria were grown in 2 ml LB medium containing ampicillin overnight at 37°C. For protein expression culture, the 2ml starter culture was added into 500 ml LB medium with ampicillin and grew at 37°C till an OD=600 nm reached 0.6. Protein expression was induced by addition of IPTG to a final concentration of 1 mM. After 4 hours gentle shaking at 25°C, bacteria were pelleted by centrifugation (6000 rpm, 15 min, at 4°C) and resuspended in lysis buffer. The suspension was sonicated by Sonopuls HD70 sonicator 3 times for 30 seconds. After 20 min incubation on ice, lysates were cleared by centrifugation (14000 rpm, 20 min, at 4°C). GST-fusion proteins in the lysates can be enriched by Glutathione Sepharose 4B and eluted by 50 mM Tris•Cl (pH 8.0) buffer containing with 20 mM glutathione. The eluted fractions were pooled, dialyzed overnight at 4°C.

Lysis buffer

1% Triton X-100

1 mM TCEP
1 mg/ml Lysozyme
1 U Benzonase
Protease inhibitor
Diluted in 50 ml PBS

Dialyze buffer
50 mM Tris•Cl (pH 8.0)
0,5 mM EGTA
10% Glycerol
100 mM K_2SO_4
2 mM $MgSO_4$

2.2.2.4 GST pull-down and peptide pull-down

GST-fusion proteins were expressed and prepared as described. Same amount of lysates containing GST-fusion proteins or purified GST-fusion proteins were incubated to couple with glutathione sepharose for 1 hour at 4°C. The beads were washed three times with triton buffer and once with ice cold TBS. Then incubate the beads coupled with GST or GST-fusion proteins with equal volume of lysates of HEK-293 cells transfected with target proteins for 30 minutes. After washing with triton buffer for 3 times, bound proteins were load on SDS gel and blotted to Nitrocellulose Transfer Membrane (Schleicher & Schuell). GST-fusion proteins were stained by Ponceau S and pulled down protein was incubated with appropriate antibody and detected by ECL or Odyssey Infrared Imaging System.

In peptide pull-down assay, streptavidin dynabeads were firstly saturated with non-modified or with R297 Citrullinated C-terminal C/EBPα peptides (2 mM) for 30min in PBS (pH 7.4). Excess peptides were washed off with PBS containing 0.1% BSA and lysis buffer. Beads were then incubated with cell extracts prepared from transfected HEK-293 cells for 1h. After washing, bound proteins were eluted with SDS–PAGE loading buffer. Western blots were incubated with appropriate antibodies and revealed by ECL.

Triton buffer

50 mM Tris•Cl pH 7.5

150 mM NaCl

1 mM EDTA

1% Triton X-100

Protease inhibitors

Lysis buffer

20mM HEPES (pH 7.8)

350mM NaCl

30mM MgCl2

1mM EDTA (pH 8)

0.1mM EGTA (pH 8)

20% glycerol

0.5% NP-40

Protease inhibitors

2.2.2.5 Co-immunoprecipitation

For co-immunoprecipitation assays, transfected HEK-293 cells were wased with PBS and then lysed in CoIP buffer. One fiftieth of the lysate was used as a control for protein expression (input). The cleared lysates were incubated with appropriated antibody (2-5 μg) or mouse IgG (negative control) for 1 hour at 4°C. Then add 15 μl Dynabeads Protein G (Invitrogen) and incubate for 1 hour at 4°C. The G-Sepharose beads bound complexes were washed three times with lysis buffer and isolated on magnet. Input samples and sepharose beads pellets were resuspended in protein loading buffer and heated for 3 min to 95°C. Samples were resolved by SDS-PAGE and immunoblotting.

CoIP buffer

50 mM Tris•Cl (pH 8)

150 mM NaCl

0.5% NP-40
1 mM EDTA
Protease inhibitors

2.2.2.6 SDS-PAGE and Immunoblotting

Proteins were separated by electrophoresis in 12% or 15% SDS-polyacrylamide gel prepared according to standard procedures (Maniatis Molecular Cloning Laboratory Manual). Proteins were then wet-blotting for 60 minutes at 80V to nitrocellulose membranes (Schleicher&Schuell). After the transfer, membranes were stained with Ponceau S or blocked with 5% non-fat milk for 30 minutes at RT. Membranes were incubated with primary antibodies diluted in blocking milk overnight at 4°C. After extensive washes in PBS-Tween, membranes were incubated with secondary antibodies conjugated to horseradish peroxidase diluted in 5% non-fat milk for 2 hours. Antigen-antibody complexes were detected by chemiluminescence (ECL system, Amersham). Alternatively, membrane was blocked by Roti-block buffer. The primary antibodies and secondary antibodies conjugated to the fluorochrome IRDyeTM (Li-Cor) were also diluted in Roti-block buffer. The signal from antibody can be detected and relatively quantified using Odyssey Infrared Imaging System and Analysis software.

Running buffer (10 x)
250 mM Tris base
2 M Glycine
35 mM SDS
Dissolve in deionized water

Transfer buffer
25 mM Tris base
190 mM glycine
20% methanol
Dissolve in deionized water

5% non-fat milk

5 g non-fat milk powder (Merck)
0.5% Tween
in 1 x PBS-Tween.
Freeze at -20°C.

Roti-Block (Roth)

Dilute 1:5 in deionized water

2.2.2.7 Nuclear extract

Transfected cells were washed in ice cold PBS and collect by centrifugation at 1200rmp for 3 minutes. Cells was resuspended in hypotonic buffer (800µl per 10 cm plate) and incubated for 5 min at 4°C. Nuclei were pelleted by centrifugation (14000 rpm, 3 min, at 4°C) and then resuspended in hypertonic buffer (100µl per 100 µl pellets). After 20 min shaking at 4°C, lysates were cleared by high-speed centrifugation (14000 rpm, 3 min, at 4°C).

Hypotonic buffer

0.2% NP-40
10% Glycerol
50 mM Tris•Cl, pH 8
10 mM KCl
1 mM EDTA
Add protease inhibitors (prior use).
Store at 4°C.

Hypertonic buffer

1% NP-40
10% Glycerol
50 mM Tris HCl, pH 8
10 mM KCl

400 mM NaCl
1 mM EDTA
Add protease inhibitors (prior use).
Store at 4°C.

2.2.2.8 *In vitro* citrullination/ deimination assay

Plasmid expressing HA tagged PADI4 were transfected into HEKT cells by $CaPO_4$ method. 48 hours post transfection, cells were washed by PBS and resuspended in lysis and immunoprecipitation buffer. After centrifugation, revmove pellet and enrich HA-PADI4 protein by immunoprecipitation with anti-HA antibody and protein G coated Dynabeads. Deimination reactions were carried out by incubating immunoprecipitated bead-bound PADI4 and 2 μg purified GST peptides at 37°C in deimination buffer for 2 hours. After reaction, samples were separated by SDS-PAGE followed by western blot with anti-modified citrulline antibody.

Lysis and immunoprecipitation buffer
20mM HEPES (pH 7.8)
150 mM NaCl
20% Glyceral
0.1mM EGTA
1mM EDTA (pH 8)
0.5% NP40
Add protease inhibitors (prior use).

Deimination buffer
50 mM Tris•Cl (pH 7.5)
150 mM NaCl
5 mM DTT
5 mM $CaCl_2$
5% Glycerol

2.2.3 Working with cells

2.2.3.1 Growth of Mammalian Cells

Human kidmeny cell HEK-293T and C/EBPα deficient mouse embryonic fibroblasts (C/EBPα-/- MEFs) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FCS. Mouse myeloid precursor 32Dcl3 cells were grown in RPMI1640 (Invitrogen) supplemented with 10% FCS in the presence of IL-3 (supplied by WEHI-3B conditioned medium). Human leukemic monocyte lymphoma U937 cell line, human promyelocytic leukemia HL-60 line and erythroleukemic K562 cell line were cultured in RPMI1640 supplemented with 10% FCS. All media were supplemented with 1% penicillin/streptomycin and cells were grown at 5% CO_2, 37°C.

2.2.3.2 Transfection with $CaPO_4$

2 x 10^6 HEK-293T were plated on a 10 cm dish. The next day, DNA solution was mixed with 450 µl TE buffer and 50 µl 2.5M $CaCl_2$. Then 2xHBS solution was added dropwise onto the DNA solution and vortex the buffer for 20 seconds. After 30 min incubation, the DNA- $CaPO_4$ solution was pipetted onto the cells.

$CaCl_2$ (2.5 M)

Dissolve 36.76 g CaCl2 in 100 ml deionized water. Filter sterilize (0.45µm filter) and store at -20°C.

2 x HBS

50 mM HEPES (pH 7.0)

250 mM NaCl

1.5 mM Na_2HPO_4 Dilute in deionized water.

Filter sterilize (0.45µm filter) and store at -20°C

2.2.3.3 Liposomal transfection

Transfection with liposomal reagents offers a higher efficiency and reproducibility. Metafectene reagent (Biontex) was used in transfecting HEK-293T cells and C/EBPα-/- MEFs. Cells were seeded 12 hours prior transfection. Per 12-well to be transfected, 0.5µl

Metafectene reagent were mixed with 37.5µl Serum free-DMEM and incubated for 15 min at RT. Then up to 0.5 µg plasmid DNA was added and mixed by piping. After 25 min incubation, the mixture was added dropwise onto the cells. Cells were harvested 48 hours post-transfection.

2.2.3.4 Electroporation

The haematopoietic cells are not easily transfected with DNA by liposomal reagent or $CaPO_4$ method. The myeloid precursor 32D and leukemia U937 cells were electroporated with Amaxa Cell Line Nucleofector device and Amaxa® Cell Line Nucleofector® Kit C (for U937) and Kit V (for 32D). 1 x 10^6 cells and 2 µg purified plasmid DNA were mixed with Nucleofector solution in the supplied cuvettes at room temperature. Then insert the cuvette with cell/DNA suspension into the Nucleofector® Cuvette Holder and selected program for transfection. After electroporation, add 1 ml culture medium to the cuvette and gently transfer the sample into the prepared 12-well plate.

2.2.3.5 Reporter assay

To examine the transcriptional activity of C/EBPα, a luciferase reporter was used containing a fragment of the cMGF promoter (-82 to -41), with two palindromic C/EBP binding sites (Sterneck et al, 1992). For examination of the transcriptional activity of E2F, a reporter containing a TATA box and 6 conserved E2F binding sites was used (Porse et al, 2001). Transfection of either 293T or MEFs was carried out in duplicates with Metafectene (Biontex) as described above. 48 hours post-transfection, cells were lysed in 100µl Triton buffer and cleared by high-speed centrifugation (12000 rpm, 10 min, 4°C). 15µl of each lysate were transfered to an 96-well plate and the luciferase activity were measured with an Berthold Lumat LB9501 plate reader luminometer. The data are representative of three independent experiments, duplicates are graphed as the mean±SD.

Lysis buffer

50 mM Tris•Cl (pH7. 5)

1 mM EDTA

1 mM DTT
1% Triton

Substrate-Reaction buffer
375µl Luciferin
250µl ATP (of 20 mM solution)
50µl $MgSO_4$ (of 1 M solution)
Dilute in 25 mM Gly-Gly.
Prepare prior use, protect from light.

2.2.3.6 Adipogenesis

Vector control, WT C/EBPα, I294A, R297A, R297Q, R297K, BRM2 were transfected by Metafectene (as described above) into C/EBPα-/- MEFs and selected with medium containing 3mg/ml puromycin (InvivoGen). After selection, stable transfectans were seeded in duplicates on tissue culture dishes and grown to confluence. Cells were differentiated with MEM-AlphaMedium supplied with 10% serum, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10µg/ml insulin and 1µM dexamethasone for 2 days. From day 3 onwards, cells were cultured in alpha-MEM, 10% serum and 10µg/ml insulin. Medium was refreshed every 2 days. 10 days post drug treatment, adipocytes were fixed with 4% paraformaldehyd and stained by Oil Red O. Cellular morphology was documented using bright-field microscopy and protein expression were determined by western blot.

Insulin (Sigma)
Dissolve 100 mg in 10 ml acidified water (add 0.1 ml glacial acetic acid). Store at -20°C.

Dexamethasone (Sigma)
Dissolve 40 mg in 10 ml ethanol (10 mM stock). Store at -20°C.

IBMX (Sigma)
Dissolve 100 mg in 9 ml ethanol (50 mM stock). Store at -20°C.

2.2.3.7 Oil-Red-O staining

Cells were washed with PBS and fixed with Roti®-Histofix 4% (Roth) for 10 min. After washed in PBS, cells were stained with Oil Red-O working solution for 15 min. Finally, cells were washed with and supplied in deionized water.

Oil-Red-O stock solution and work solution

300 mg Oil-Red-O (Sigma) dissolved in 100 ml isopropanol and store at RT (protect from light). Oil-Red-O working solution should be prepare prior use: Mix 3 part Oil-Red-O stock solution with 2 parts deionized water. Let stand for 10 min and filter through a folded Whatman filter paper (Schle-icher&Schuell).

2.2.3.8 Cell growth assay and Crystal violet staining

C/EBPα -/- MEFs were tranfected and grown under puromycin selection. 1 x 10^5 stable transfectans were seeded in triplicates on tissue culture dishes and grown for 6 days. The conlonies were stained with crystal violet for 10 min and extensive washed with deionized water.

Crystal violet

0.1% (w/v) crystal violet dissolved in 10% ethanol, filter through a folded Whatman filter paper (Schle-icher&Schuell). Store at RT (protect from light).

2.2.3.9 Chromatin Immunoprecipitation (ChIP) analysis

Harvest C/EBPα -/- MEFs cells that are induced adipogenesis by WT C/EBPα or mutants, resuspend in 10 ml fresh DMEM/RPMI, cross-link with 270 µl of 37 % formaldehyde (1% final concentration). After incubation for 15 min at room temperature with modest shaking, stop crosslink by adding glycine to a final concentration of 0.125 M (1 ml of 1.375 M). Centrifuge in swinging bucket at 1000 rpm (15 ml greiner tube), 4°C, 2 min, decant supernatant, wash with ice cold PBS. Add Protease Inhibitors (from pellet) and 20 µl NaButyrate (0.5 M stock, optional) to 1 ml lysis buffer. Lyse cells in 400 ul Cell Lysis Buffer incubate 10 min on ice, vortex. Spin down at 1000 rpm in spinning bucket rotor, 4°C, 5 min.

Resuspend pellet in 400 µl Nuclear Lysis Buffer and incubate on ice for 10 min and then add 600 µl Nuclear Lysis Buffer. Sonicate in 15 ml tube in Bioruptor at high intensity with 0.5 interval for 8 min. Centrifuge for 10 min at full speed, transfer to new Eppendorf tube and repeat 2 more times. Divide into different tubes 200 µl each and add 400 µl IP Dilution Buffer. Add 10 µl of magnetic Protein G beads incubate for 0.5 h to pre-clear the samples. Remove beads with magnet and transfer supernatant to fresh tube, add various amounts of antibody (usually 1-5 µg), save one tube for input. If more samples are required, the input can be taken from lysate supernatant after IP.

Block Dynabeads Protein G (Dynabeads Protein G, cat. no. 100.04D, Invitrogen) with 500 µg/ml tRNA for 0.5 h. Wash blocked beads twice with IP dilution buffer. Add 10 µl of blocked Dynabeads Protein G to samples for 3 hours in 4°C. Apply magnet, invert several times to collect all magnetic beads. Wash with wash buffer I, II and III. Elute with 150 µl elution buffer at room temperature. Mix well, vortex, spin down, apply magnet and transfer supernatant to new tube, repeat and combine the elution fractions.

Add 1 µl RNaseA (1 mg/ml stock), 18 µl 5 M NaCl (final concentration 0.3 M) to reverse crosslink of samples and input incubate at 67°C overnight. Add Proteinase K, 6 µl (10 mg/ml, stock) to samples, 3 µl to input, incubate at 45°C for 2 h. Isolate DNA with QIAquick Gel Extraction Kit (Qiagen) following manufacturer's specifications. Resuspend product in 30 µl RNase/DNase-free water, resuspend input in 66.7 µl. Use 1 µl DNA from ChIP samples, 1 µl input (1:10 dilution) for real time quantitative PCR reaction. Primers for PCR are listed in Table.2.3.

Cell Lysis Buffer

10 mM Tris•Cl pH 8

10 mM NaCl

0.2 % NP-40

Add protease inhibitors (prior use).

Nuclei Lysis Buffer

50 mM Tris•Cl pH 8

10 mM EDTA

1 % SDS

Add protease inhibitors (prior use).

IP Dilution Buffer

20 mM Tris•Cl pH 8

2 mM EDTA

150 mM NaCl

1 % Triton X-100

0.01 % SDS

Add protease inhibitors (prior use).

IP Wash I

20 mM Tris•Cl pH 8

2 mM EDTA

50 mM NaCl

1 % Triton X-100

0.1 % SDS

Add protease inhibitors (prior use).

IP Wash II

10 mM Tris pH 8

1 mM EDTA

0.25 M LiCl

1 % NP 40

1 % Deoxycholic Acid (Sodium Salt)

IP Wash III

20 mM Tris pH 7.6

50 mM NaCl

Elution Buffer

100 mM Na_2HCO_3

1 % SDS

2.2.3.10 RNA Extraction and cDNA Synthesis

RNA from mammalian blood cells or adipocytes was prepared using the High Pure RNA Isolation Kit (Roche) following the manufacturer's instructions. First strand cDNA synthesis is performed with SuperScript™ II Reverse Transcriptase kit (invitrogen). Briefly, 500ng mRNA is mixed with 1 µl Oligo (dT) (500 µg/ml), 1 µl dNTPp and 10 µl water. Heat the mixture to 65°C for 5 min and quick chill on ice. Then add 5 x First-Strand Buffer, 1 µl DTT (0.1M) and 1 µl RNaseOUT™. Incubate the mixture at 42 °C for 2 min. Add 1 µl SuperScript™ II Reverse Transcriptase and mix by pipetting gently. Incubate the mixture at 42 °C for 50 min followed by heating at 70°C for 15 min. The cDNA can be used as template for amplification in Real time quantitative PCR.

2.2.3.11 Real time quantitative PCR

Real time quantitative PCR (qPCR) was performed on an ABI Prism 7000 (Applied Biosystems) using SYBR green PCR master mix 7000 (Applied Biosystems) according to the manufacturer's instructions. The relative RNA expression levels were calculated by using the comparative threshold cycle (C_T) method, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression values were used to normalize the analyzed RNA levels. ChIP DNA levels were calculated by using the comparative C_T method, normalized to input, and expressed as anti-C/EBPα versus IgG. The sequences of the primer pairs for qPCR are listed in Table.2.3

2.2.3.12 Small interference RNA (siRNA)

RNA interference is a technology that bases on the finding that a 20-mer double-stranded RNA oligonucleotide, namely small interference RNA (siRNA), can target mRNA for degradation and allow gene silencing (Elbashir et al, 2001). The siRNA can be

qPCR target	PCR Primers used
maP2 mRNA forward	5’ caa aat gtg tga tgc ctt tgt g 3’
maP2 mRNA reverse	5’ ctc ttc ctt tgg ctc atg cc 3’
mPPARγ mRNA forward	5’ gca tgg tgc ctt cgc tga tgc 3’
mPPARγ mRNA reverse	5’ tac gtt tat ctg gtg ttt cat 3’
mAdipsin mRNA forward	5’ gct atc cca gaa tgc cct cgt t 3’
mAdipsin mRNA reverse	5’ cca ctt ctt tgt cct cgt att gc 3’
mGAPDH mRNA forward	5’ aat gtg tcc gtc gtg gat ctg a 3’
mGAPDH mRNA reverse	5’ gat gcc tgc ttc acc acc ttc t 3’
mPADI4 mRNA forward	5’ cag cct ctc cag gag tca tc 3’
mPADI4 mRNA reverse	5’ tag atc agg gct tgg act gg 3’
hPADI4 mRNA forward	5’ cga aga ccc cca agg act 3’
hPADI4 mRNA reverse	5’ agg aca gtt tgc ccc cgt g 3’
hGAPDH mRNA forward	5’ cat gtt cca ata tga ttc cac 3’
hGAPDH mRNA reverse	5’ cct gga aga tgg tga tg 3’
mPPARγ ChIP forward	5’ ttc aga tgt gtg att agg ag 3’
mPPARγ ChIP reverse	5’ aga cttg gta cat tac aag g 3’
maP2 ChIP forward	5’ cct cca caa tga ggc aaa tc 3’
maP2 ChIP reverse	5’ ctg aag tcc aga tag ctc 3’
mDHFR ChIP forward	5’ gca ttg cag tgt gca gaa gag c 3’
mDHFR ChIP reverse	5’ cag cgg gga taa aat cct acc agc 3’

Table 2.3 Primers used for qPCR. m=mouse, h=human.
mRNA: for transcript; ChIP: for Chromatin Immunoprecipitation.

synthesized and alternatively expressed by vectors as short hairpin RNAs (shRNAs). In this study, psiRNA-7SKGFPzeo vector (InvivoGen) was applied to generate shRNA. This vector contains RNA polymerase III promoter to transcribe shRNAs and a Zeocin selection marker fused to an internal ribosome entry site (IRES) GFP for selection or sorting. The expressed hairpin RNA contained two complementary sequences of 21 nucleotides, homologous to a segment of the gene of interest, separated by a short region

of 5-9 nucleotides. shRNA oligos against mouse PADI4 or human PADI4 were designed using InvivoGen siRNA Wizard program (http://www.sirnawizard.com/design.php) and subjected to BLAST search (http://www.ncbi.nih.gov/BLAST/) to exclude homology to any additional known sequence. A scrambled shRNA sequence was used as control.
Double-stranded DNA oligonucleotides were synthesized and ligated into the BbsI site of the psiRNA construct. Then E.coli GT116 stain was used to transform and grown the ligated plasmid. Since this site is flanking a bacterial lacZ α-peptide cassette, successful ligation of the oligonucleotides destroys the lacZ gene. Thus, growth on X-Gal plates allows discrimination between parental clones (blue) and insert-containing white clones. The psiRNA-7SKGFPzeo vector containing an siRNA construct generated 3 bands after SpeI digestion: 1644bp, 1206bp and 741bp and further confirmed by sequencing. Sequences targeted by shRNAs are listed in Table. 2.4

2.2.3.13 Fluorescence activated cell sorter (FACS)

The IRES GFP contained in the psiRNA-7SKGFPzeo vector allowed sorting of the transfected cells. Electroporated 32D or U937 cells were washed with FACS buffer and centrifuge at 1200 rpm for 5 min (can be at RT). In order to get a single cell suspension, cells were passed through a cell strainer (BD Bioscience). Each tube receives 100 ml of the corresponding cell suspension at a density of 0.5 x 10^6 cells/ 100 µl. Add 1 µl of CD11b-PE (BD Bioscience) antibody to cell suspension, vortex quickly and carefully. Incubate for 20 min at 4°C in the dark. Then add 3 ml of FACS buffer into cell suspension and centrifuge as above, decant carefully the supernatant. Resuspend the cells in 300 µl FACS buffer and vortex carefully. The sorting and analysis of the cells were performed on ACCURI cytometers (BD Bioscience).

FACS buffer:
PBS
2mM EDTA
2% FCS
Filter before each experiment onto 0,45 m filter. Store at 4°C.

Cloning for ShRNA	Oligonucleotide used for cloning
Scramble sense	5’ acc tcg tcc atc gaa ctc agt agc ttc aag aga gct act gag ttc gat gga ctt 3’
Scrambel antisense	5’ caa aaa gtc cat cga act cag tag ctc tct tga agc tac tga gtt cga tgg acg 3’
Human PADI4 sense 1	5’ acc tcg cac aac atg gac ttc tac gtt caa gag acg tag aag tcc atg ttg tgc tt 3’
Human PADI4 antisense 1	5’ caa aaa gca caa cat gga ctt cta cgt ctc ttg aac gta gaa gtc cat gtt gtg cg 3’
Human PADI4 sense 2	5’ acc tcg agg tgt acg cgt gca gta ttt caa gag aat act gca cgc gta cac ctc tt 3’
Human PADI4 antisense 2	5’ caa aaa gag gtg tac gcg tgc agt att ctc ttg aaa tac tgc acg cgt ac acct cg 3’
Mouse PADI4 sense	5’ acc tcg cac ctt cat caa cga ctt ctt caa gag aga agt cgt tga tga agg tgc tt 3’
Mouse PADI4 antisense	5’ caa aaa gca cct tca tca acg act tct ctc ttg aag aag tcg ttg atg aag gtg cg 3’

Table. 2.4: Oligonucleotides used for cloning siRNA expression constructs. Sense and antisense oligonucleotides were annealed and inserted in the BbsI site of the psiRNA-7SKGFPzeo (InvivoGen) construct.

3 Results

3.1 Working hypothesis

We have recently shown that C/EBPβ is extensively regulated by modifications on arginine and lysine side chains (Kowenz-Leutz et al, 2010; Pless et al, 2008). These post-translational modifications (PTM) crosstalk to each other to alter transregulatory functions on C/EBPβ. This raises the possibility that such regulatory mechanism also occurs on C/EBPα, another member of CCAAT enhancer binding protein family. Here, we focus on detecting PTMs throughout C/EBPα and trying to understand how they cooperate or antagonize to regulate C/EBPα activities. Of particular importance is the question whether certain specific PTMs effect C/EBPα mediated cell cycle control and differentiation, and whether such PTMs may be involved in the development of leukemia.

3.2 Post-translational modifications detected on C/EBPα

C/EBPα,β are dynamically regulated by post-translational modifications including phosphorylation and methylation (Johnson, 2005; Kowenz-Leutz et al, 2010; Lee et al, 2010; Pless et al, 2008). To examine the possibility that additional modifications also occurs on C/EBPα, we performed protein purification and mass spectrometric analysis. A 8× Histidine tag was placed at the N-terminus of full length rat C/EBPα, and the protein was expressed in HEK-293T cells. Whole-cell lysates were prepared with guanidine hydrochloride buffer. Afterwards the histidine-tagged C/EBPα protein in the lysates was enriched by Ni-NTA affinity purification. C/EBPα fractions were eluted from Ni-NTA by an imidazole gradient and further purified by reversed phase HPLC (purification process is shown in Fig. 3.1A, details in Materials and Methods 2.2.2.1). Mass spectrometry analysis shows that mono-, dimethylation, as well as citrullination occur on several conserved arginines ((Fig. 3.1D). Morover, phosphorylations are detected on serine 16, 17, 21, 27, 119, 277 and 282. Interestingly, lysine 159 (also SUMOylation site) and lysine 352 (Leucine Zipper **d** position) are found to be mono-methylated.

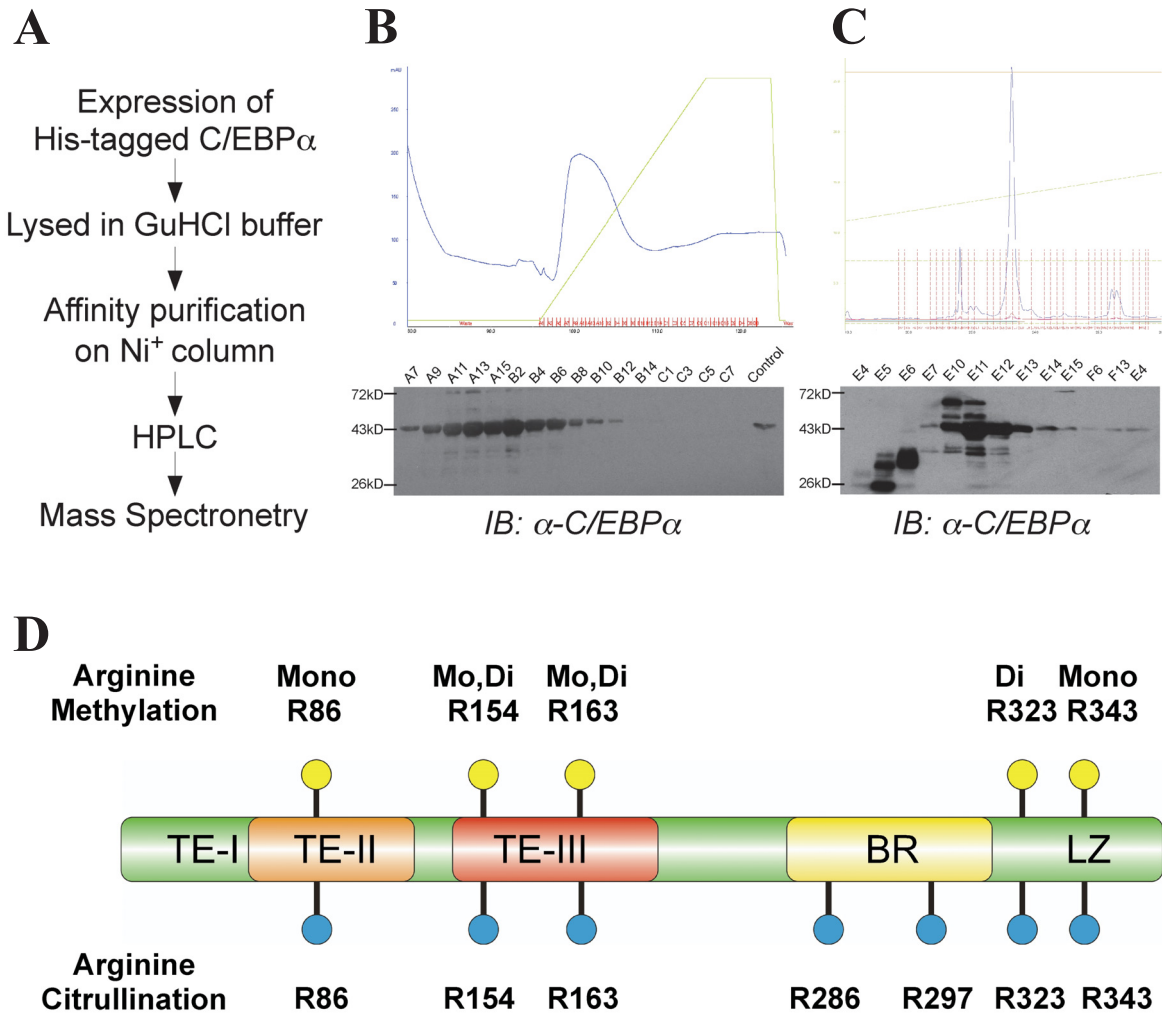

Figure 3.1: Purification and post-translational modifications detected on C/EBPα. A) Scheme of protein purification process. His-tagged C/EBPα was transected into HEK293T cells and lysed under denaturing conditions followed by affinity purification and reverse phage chromatography. B) His-tagged C/EBPα protein is enriched by affinity to nickel chelating resin and eluted by buffer with gradient concentration of imidazol; Blot shows purified C/EBPα from by elution fractions. C) C/EBPα is further purified by reversed phase HPLC; Blot shows further purified fractions of C/EBPα. D) Schematic representation of PTMs that are identified on C/EBPα by mass spectrometry analysis. Citrullination, Mono-methylation (Mo) and Di-methylation (Di) that occur on C/EBPα arginines are indicated.

These data indicate that, in addition to reported phsophorylation and SUMOylation, more PTMs including methylation and citrullination also occur on the transcription factor

C/EBPα. It is also of particular interest that in the regulatory domain and in the leucine zipper, more than one modifications were found to occur on the same residue (such as R154, R163, K159). This suggests that such modifications may regulate different functions of C/EBPα.

3.3 Deimination/Citrullination on C/EBPα and functional consequence

3.3.1 Citrullination on basic region R297 impairs DNA binding

Among those PTMs that are detected on C/EBPα, citrullination in the basic region residue R286 and R297 was of particular interest. Crystal structure shows that side chains of these two arginines form like arms to stabilize C/EBPα on the DNA major groove (Fig. 3.2B). Nevertheless, R297 was shown to be a critical residue for C/EBPα function: mutation of this residue (R297P) has been found in a human patient diagnosed with a AML M2 subtype (Benthaus et al, 2008) and experimental mutation (R297A) was involved in pathological myeloproliferation as in the C/EBPα BRM2 mutant (Fig. 3.2A). This raised the questions whether R297 citrullination regulates C/EBPα functions. The structural data indicated that the guanidinium group of R297 in the C/EBPα bZIP is exposed to the solvent and may potentially interact with accessory proteins, in addition to an interaction that might occurs with the phosphate group of G1 in its cognate DNA binding site (Fig. 3.2B) (Miller et al, 2003; Tahirov et al, 2001). The situation is somewhat complicated by the fact that BRM2 contained a second mutation at I294A, yet, the biological defect of BRM2 might be attributed to either I294A or R297A single mutation. Thus, to begin with, we asked whether the citrullination of R297 and any mutation in BRM2 altered the interaction between C/EBPα with DNA or with protein partners.

To examine these possibilities, we first determined the interaction between C/EBPα peptide and DNA binding sites. We employed chemically synthesized C/EBPα bZIP peptides (residue 278 to 340) to rule out any complications that may occur by additional modifications if proteins are produced in eukaryotic cells, bacteria, or in vitro translation cocktails. We then measured the affinity of the C/EBPα peptides-DNA interaction using

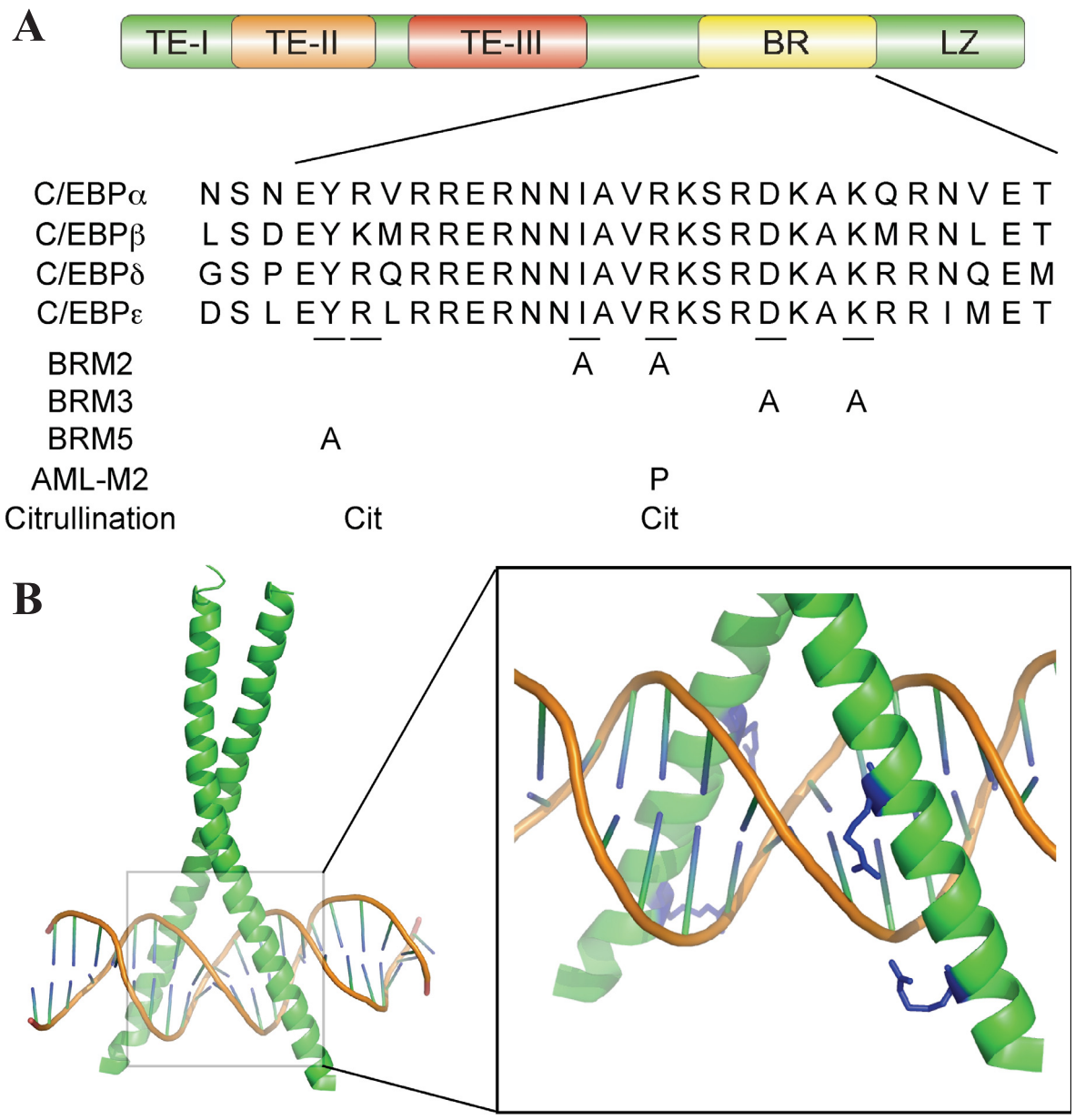

Figure 3.2: Structure and sequence of C/EBPα basic region. A) Schematic representation and sequence of C/EBPα basic region. Basic-Region Mutants (BRMs), leukemia mutant on R297 and citrullination targets (R286 and R297) are indicated below. B) Left panel: crystal structure of C/EBPα basic region leucine zipper on DNA binding site. Right panel: detailed structure of basic region suggests that R286 and R297 side chains interact with DNA backbone and contribute to DNA binding affinity. The side chains of R286 (lower) and R297 (upper) are indicated in blue. Figures are generated by PYMOL software coordinating structure factors (code 1NWQ) that have been deposited in the Protein Data Bank (http://www.rcsb.org/). (Miller et al, 2003)

isothermal titration calorimetry (ITC). By ITC measurement, we can precisely monitor the enthalpy release upon the peptide-DNA interaction and calculate dissociation constant (Kd) by non-linear regression. We found that WT C/EBPα bZIP peptide binds to the DNA consensus with a Kd of 584.8 ± 188.1 nM while the R297Cit peptides showed decreased binding ability with a Kd of 990.1 ± 156.9 Nm (Table 3.1). This result suggests that citrullination on C/EBPα R297 diminishes the affinity of C/EBPα dimmers to its DNA binding site.

3.3.2 The positive charge of R297 is critical for protein-DNA complex stability

The impaired DNA affinity by citrullinated C/EBPα bZIP peptide verifies an important role of R297 that was implied by structural analysis (Miller et al, 2003). Theoretically, R297 mediates C/EBPα association with DNA backbone by its side chain guanidinium group with basic property. Since the main feature of citrullination is removing the positive charge and imine group from arginine, it is not surprising that the impaired DNA binding is potentially due to the loss of the positive charge. Moreover, our previous research pointed out that BRM2 showed abnormality in DNA binding (Zaragoza et al, 2010). We then asked if the defect of BRM2 is due to the mutation R297A, which is also a basic-neutral amino acid exchange. To distinguish more clearly between the BRM2 I294 and R297 mutations, additional mutants were generated, including separated I294A and R297A, and additional R297 substitutions to glutamine (R297Q, to partially mimic citrullination) or lysine (R297K, to retain the positive charge and resistance to citrullination). Then we investigated how residue 297 affects DNA binding by ITC measurements with bacterial expressed WT and mutated C/EBPα bZIP proteins. The results showed that R297A, R297Q, and BRM2 displayed diminished DNA binding, whereas I294A displayed stronger binding to cognate sites (Fig 3.3A and Table 3.1). These data suggest that with respect to the BRM2 double mutant binding activity represents an intermediate of decreased (R297A) and enhanced (I294A) DNA-binding affinity. The R297K mutant that retains the positive charge also displays recovered or even stronger DNA binding. These results are consistent with the ITC data from synthesized WT and R297Cit peptides and further confirmed our hypothesis that the positive charge of residue 297 contributed to C/EBPα-DNA interaction.

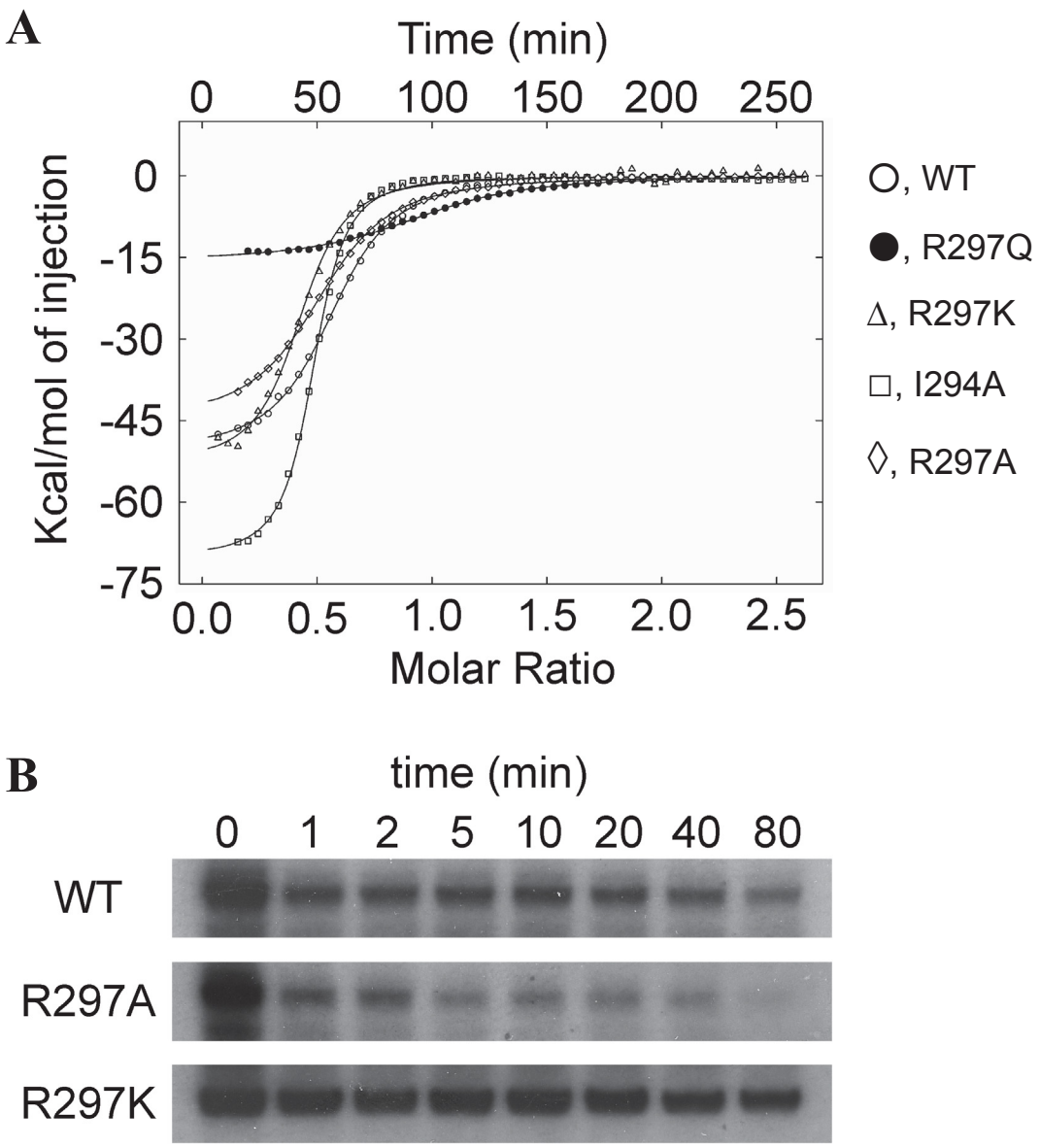

Figure 3.3: R297 mediated C/EBPα DNA binding. A) Differential DNA binding affinity of C/EBPα bZIP proteins by ITC measurement. The panels show the integrated heat data for each mutant respectively: ○, WT; ●, R297Q; Δ,R297K; □,I294A; ◊, R297A. Dissociation constant (Kd) values derived from these measurements correspond to 1/K (K, association constant) are shown in Table 3.1. B) Off-rate EMSA showing the stability of C/EBPα-DNA complex. Binding reactions with WT C/EBPα or mutants were prepared with radiolabeled probes and then incubated with 100-fold cold C/EBP probes. Reaction mixtures were loaded onto a gel at regular time intervals between 1min and 80 min after addition of the unlabeled oligonucleotide. Protein-DNA complex become undetectable on the gel as they disassociate from labeled to the unlabeled oligonucleotide. Incubation time with cold probes is indicated.

C/EBPα bZIP protein	Kd (nM)
Syn-Unmodified	584.8 ± 188.1
Syn-R297 Citrulline	990.1 ± 156.9
WT	680.3 ± 22.2
I294A	221.2 ± 10.3
R297A	980.4 ± 25.3
R297Q	1302.1 ± 54.3
R297K	497.5 ± 34.7

Table 3.1. Analyzing various C/EBPα peptides in terms of their DNA-binding capacity using isothermal titration calorimetry. Synthesized biotinylated C/EBPα bZIP peptides and bacterial expressed bZIP proteins are tested. Dissociation constant (Kd) values derived from measurements in Fig.3.3 correspond to 1/K (K, association constant).

We next performed gel shift to assess off-rates by incubating binding reactions with a 100-fold excess of unlabeled oligonucleotide for different times. As is shown in Fig. 3.3B, the WT C/EBPα or R297K mutant formed stable complexes whereas dissociation of the R297A mutant from its binding site was seen within 1 min indicating a high off-rate and instability of the protein-DNA complex. Our data presented here suggest that R297 contributed to DNA binding and the positive charge of this residue stabilized protein-DNA complex.

3.3.3 R297 mediated protein interaction

In addition to DNA binding, the C/EBPα basic region was shown to mediate protein-protein interactions with other transcription factors including E2F/DP and NF-κB p50 (Paz-Priel et al, 2005; Paz-Priel et al, 2009; Zaragoza et al, 2010). Cirullination may also change protein functions by altering intramolecular folding and protein-protein interactions (Vossenaar et al, 2003). To address whether R297 citrullination changes the interaction with partner proteins, we performed peptides pull downs with chemically synthesized C/EBPα bZIP peptides that are biotin-labeled. HEK-293T cells were transfected with different constructs expressing C/EBPα, HA-E2F1, HA-DP1, Flag- NF-κB p50. Pull-down assays with these transfected cell lysates were then performed to

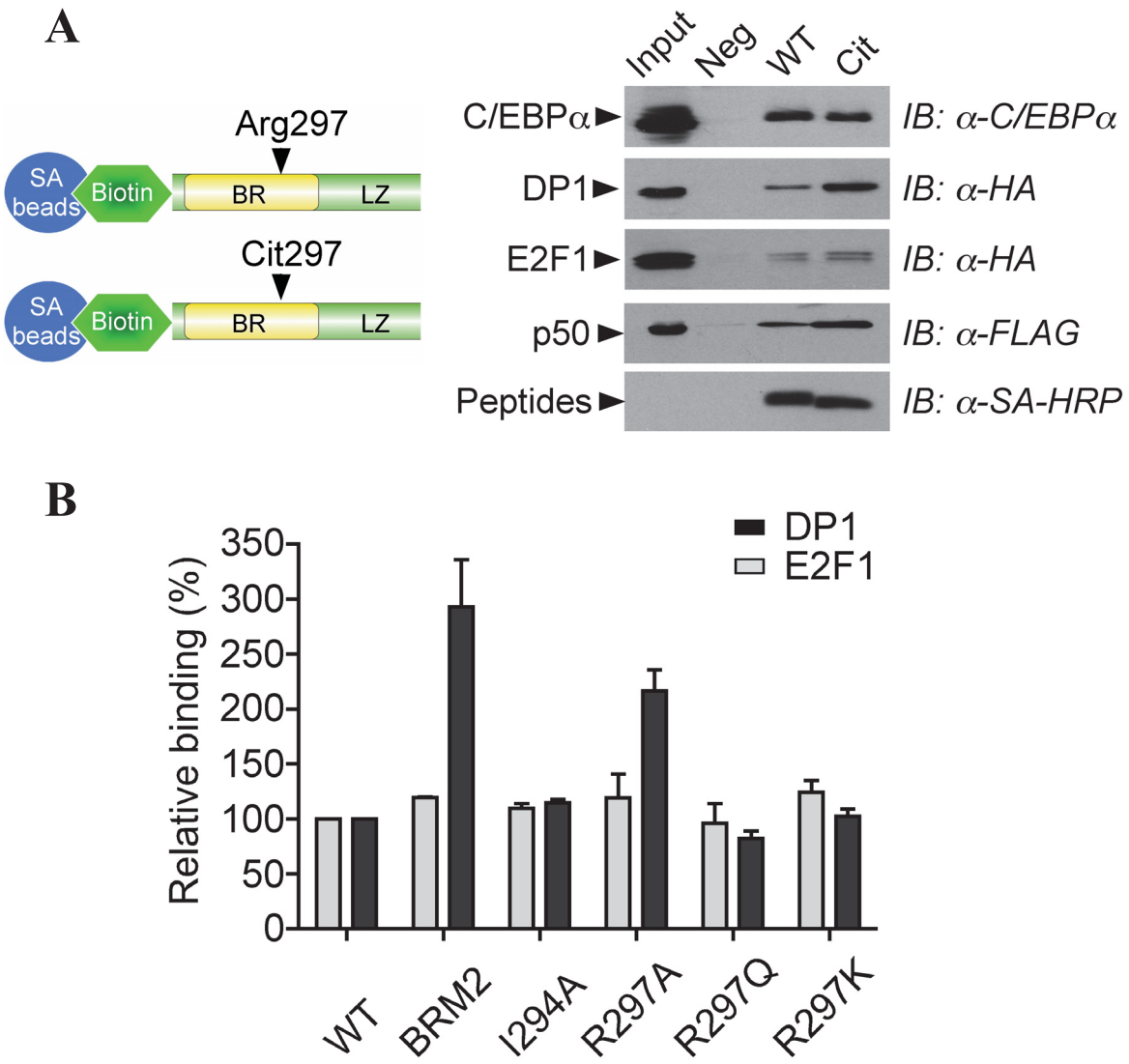

Figure 3.4: C/EBPα R297 mediated partner interaction. A) Left panel: Schematic representation of peptide pull down. Right panel: peptides with covalently attached C-terminal biotin (1mM) were bound to streptavidin dynabeads and incubated with lysates of transfected cell. Bound C/EBPα, HA-E2F1, HA-DP1 and Flag-NF-κB p50 were detected by immunoblotting and ECL with antibodies as indicated.

B) Interaction of C/EBPα to E2F1 and DP1. WT C/EBPα or mutants were expressed in HEK293T cells and the lysates were incubated with GST-E2F1 or GST-DP1. Bound C/EBPα were examined by immunoblotting and quantified by Odyssey Infrared Imaging System. Error bars indicate the means ±SD of three individual experiments.

determine differential interactions between potential partners and WT peptide versus R297Cit peptide. As is shown in Fig.3.4A, WT and R297Cit peptides bound to C/EBPα at similar level. However, R297Cit peptide displayed enhanced interactions with E2F1, DP1 and with NF-κB p50. These data suggest that citrullination of C/EBPα R297 partially impairs DNA binding and alters interaction with other proteins. The effect of enhanced binding to its interaction partners still needs to be explored. It was reported that the BRM2 mutants showed altered interaction with E2F1 or DP1 (Keeshan et al, 2003; Porse et al, 2001; Zaragoza et al, 2010). We then perfomed GST pull-down to test how C/EBPα variants interact with E2F1 and DP1. C/EBPα mutants were individually transfected into HEK-293T cells and the lysates were pulled down by GST-E2F1 or GST-DP1. Bound proteins were detected by immunoblotting and quantified by Odyssey Infrared Imaging System. As is shown in Fig. 3.4B, all C/EBPα variants interact with E2F1 as well as DP1. Similar to BRM2, R297A displays increased binding to DP1. In accordance with previous studies, however, none of these mutants show impaired interaction with E2F1 or DP1 (Keeshan et al, 2003; Zaragoza et al, 2010).

3.3.4 R297 regulates C/EBPα activity and interplay with E2F1-DP1

Several studies proposed that the balance between C/EBPα and E2F-DP critically controls precursor cell proliferation and C/EBPα mediated differentiation (D'Alo et al, 2003; Johansen et al, 2001; Porse et al, 2001). Our previous work demonstrated that E2F-DP complexes repress C/EBPα during adipocyte differentiation and that BRM2 is more sensitive to inhibition by enhanced interaction with E2F-DP (Zaragoza et al, 2010). As shown in Fig. 5B, all the mutants retain their transactivation potential in the C/EBP responsive luciferase reporter assay. However, BRM2, R297A and R297Q were strongly inhibited by E2F1-DP1, as compared to WT C/EBPα. Although I294A and R297K showed somewhat weaker transactivation, they remained partially resistant to E2F1-DP1 mediated repression. As shown in Fig. 3.5C, all mutants were expressed at similar amounts and showed similar stability compared to WT. C/EBPα induces proliferation arrest by repression of E2F-regulated S-phase genes and C/EBPα mutations in the basic region (BRM2, BRM5) failed to inhibit E2F mediated transcription (Johansen et al, 2001; Porse et al, 2001). We therefore compared trans-repression of the C/EBPα bZIP mutants

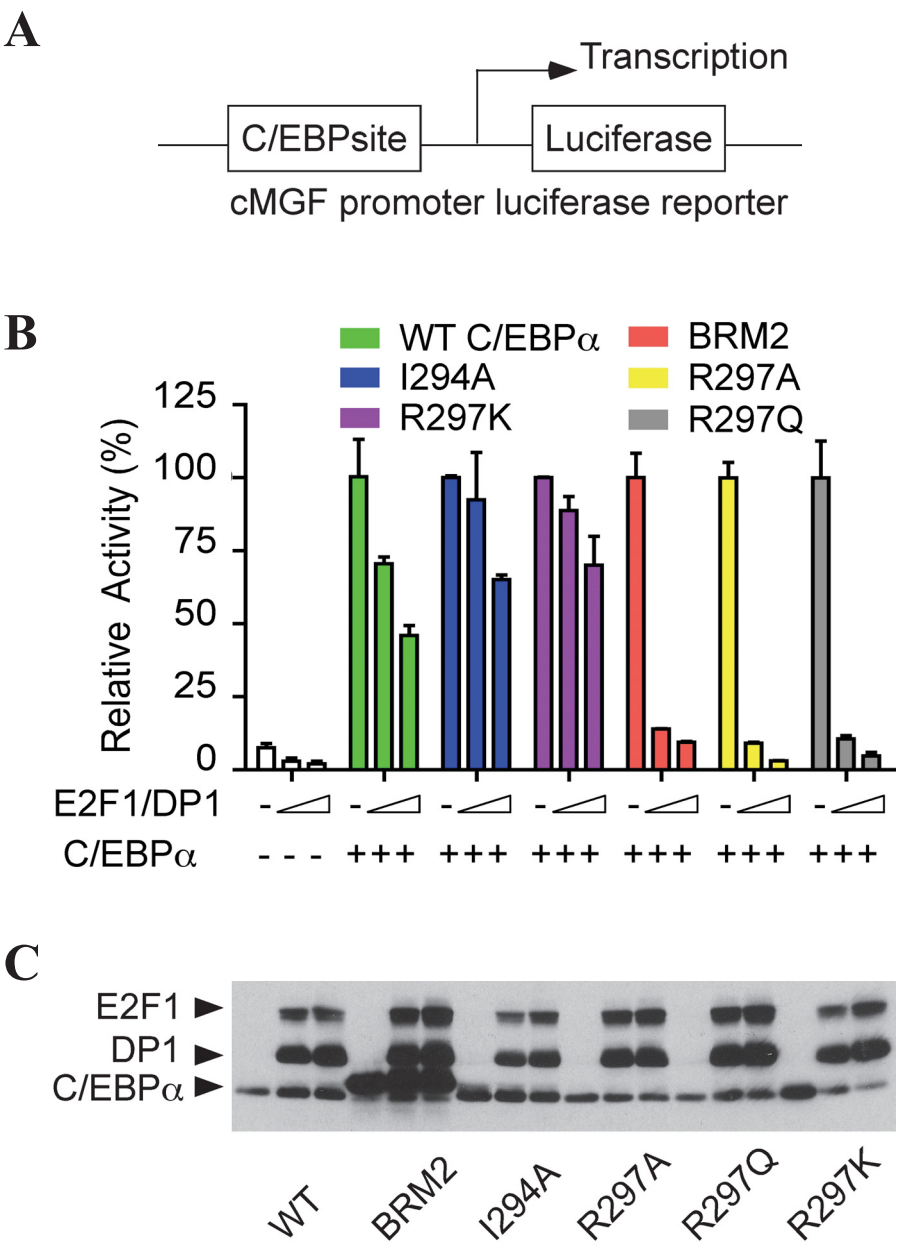

Figure 3.5: E2F-DP complex mediated repression on C/EBPα. A) Schematic representation of C/EBP responsive chicken monocyte growth factor (cMGF) promoter reporter construct. B) Differential transcriptional activity of C/EBPα variants. HEK293T cells were transfected with a C/EBP-responsive gene reporter and with plasmid expressing C/EBPα WT, BRM2, I294A, R297A, R297Q, R297K (100 ng) in the absence or presence of various amount of HA-E2F1/HA-DP1 (50 ng each, 100ng each). Error bars indicate the means ±SD of three individual experiments. C) Protein expression in B) was analyzed by immunoblotting.

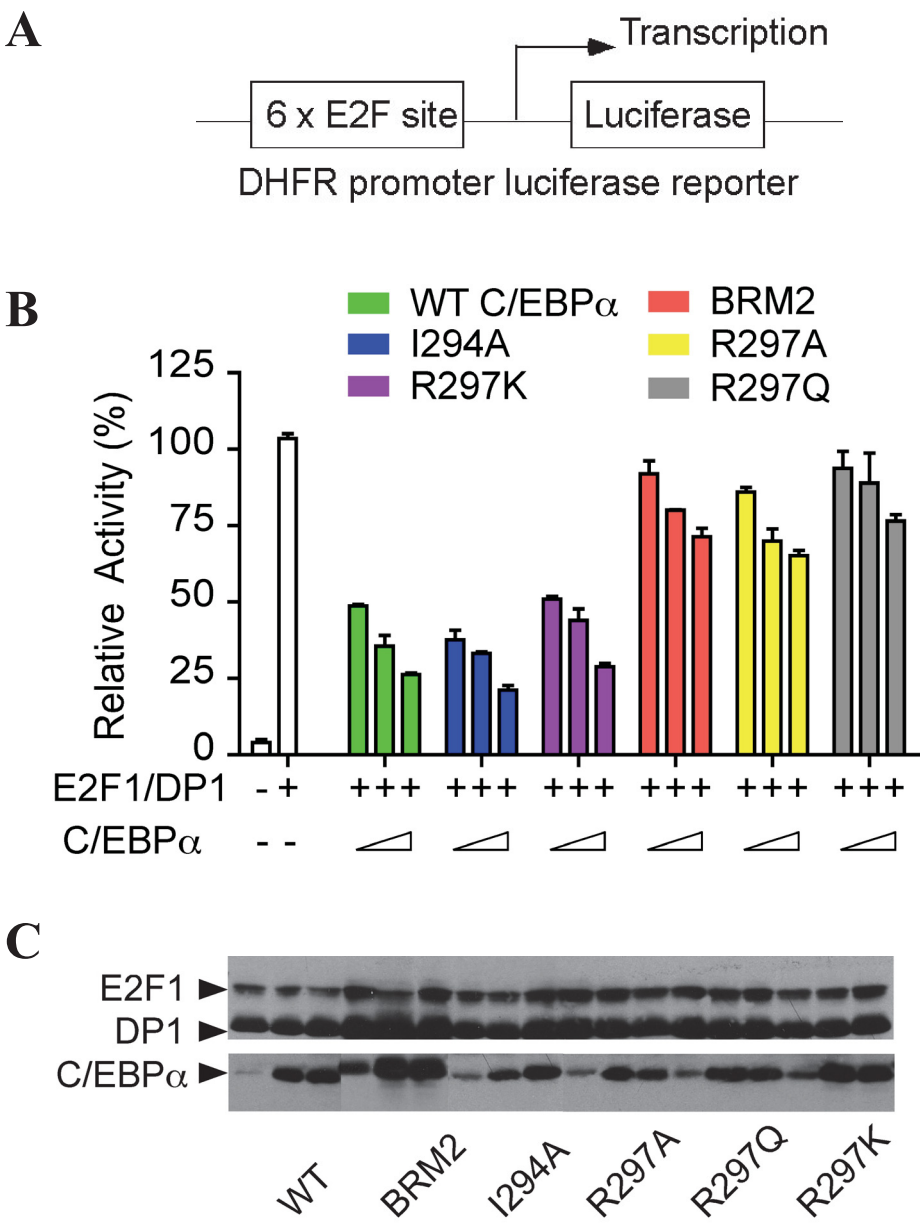

Figure 3.6: C/EBPα mediated repression on E2F-DP complex. A) Schematic representation of E2F-responsive luciferase gene reporter construct. B) C/EBPα variants show different ability to inhibit E2F-DP mediated transcription. HEK293T cells were transfected with E2F-responsive luciferase gene reporter (100 ng) and E2F1/DP1 (50 ng each), in combination with increasing amount (30 ng, 60ng, 100 ng) of constructs expressing WT C/EBPα or mutants as indicated. Error bars indicate the means ±SD of three individual experiments. C) Protein expression in B) was analyzed by immunoblotting.

on E2F dependent pE2F×6-TATA-Luciferase reporter expression. As is shown in Fig. 3.6B, WT C/EBPα, I294A and R297K are able to repress E2F efficiently, whereas BRM2, R297A, and R297Q failed to repress E2F-DP. Fig. 3.6C showed proteins expression control. These data suggest that R297 is a critical residue involved in E2F1-DP1 repression that has also been found mutated in human leukemia. Furthermore, when the positive charge on residue 297 is removed, impaired C/EBPα activities are observed and correlate with altered DNA binding ability (Fig. 3.3 and Table 3.1). In contrast, when arginine 297 is converted to lysine, the mutant protein retained WT properties in the E2F-DP interplay on C/EBP sites and on E2F sites. In accordance with earlier reports (Keeshan et al, 2003; Zaragoza et al, 2010), GST pull-down demonstrated that the C/EBPα and all the mutants interacted with E2F1 and DP1 (Fig.3.4B). We further found that distinct I294A, R297Q and R297K mutants showed similar level of binding to E2F/DP as compared to WT C/EBPα. These data suggest that, although E2F/DP complex binds to and represses C/EBPα, altered C/EBPα-E2F/DP association is not a decisive factor to inactivate C/EBPα. Rather, the reason lies in the changed DNA affinity of mutants. Taken together with the data derived from luciferase assays, we propose that the positive charge of R297 is critical to protect C/EBPα from E2F1-DP1 mediated inactivation and the susceptibility of mutants to E2F1-DP1 is mainly due to attenuated protein-DNA stability.

3.3.5 C/EBPα mediated adipogenesis and cell growth

C/EBPα regulates adipocyte differentiation in conjunction with peroxisome proliferators activated receptor γ (PPARγ) (Darlington et al, 1998; Wu et al, 1999) to collaboratively activate fat cell specific genes, such as insulin-stimulated glucose transporter (GLUT4), or fatty acid binding protein (aP2). C/EBPα deficient mouse embryo fibroblasts (C/EBPα-/-MEFs) show defective response to hormonal adipogeneic stimulation and fail to undergo complete fat cell differentiation (Darlington et al, 1998; Wu et al, 1999). C/EBPα-/-MEF cells were therefore employed in complementation type analysis of C/EBPα mutants (Wang et al, 1995). We transfected WT C/EBP α and mutants into C/EBPα-/-MEFs and selected with puromycin to obtained stable transfectans. Then

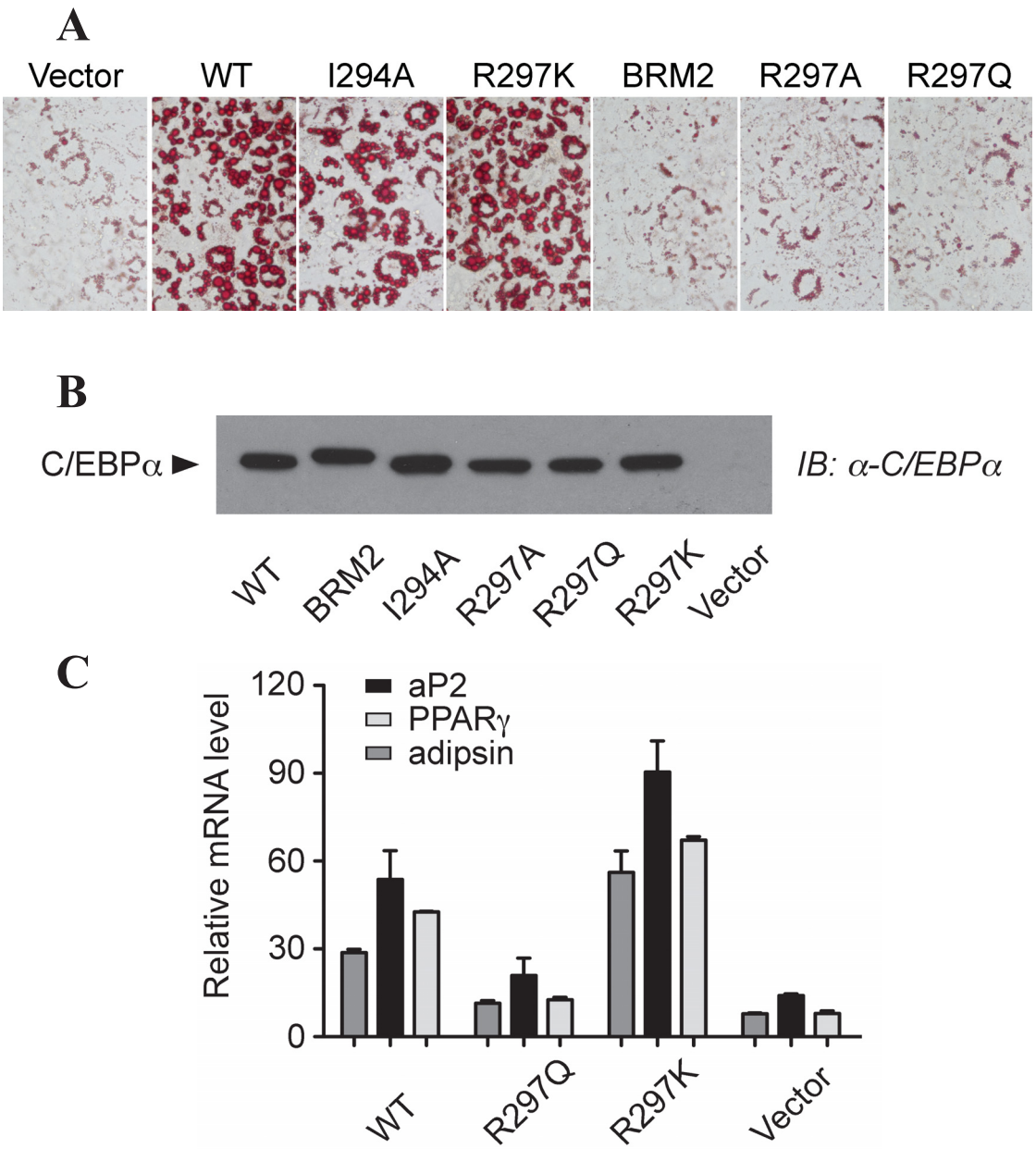

Figure 3.7: C/EBPα mediated adipogenesis. A) C/EBPα induced adipogenesis. C/EBPα-/- MEFs were stably transfected with WT C/EBPα or mutants. After selection, confluent stable transfected cells were treated with insulin/IBMX/DEX for 3 days and then cultured in medium supplemented with insulin. After 8 days induction, cells were stained by Oil-Red-O when morphological change of adipocytes can be observed. B) C/EBPα expression control in A). C) Adipogenic gene activation. Bar graphs represent transcript levels of adipogenic genes that are normalized to GAPDH. All mRNA expression analysis were performed with cells harvest after 96 h treatment with adipogenic stimulation cocktail. Error bars indicate the means ±SD of three individual experiments.

adipogenesis was induced by hormone cocktail containing 3-isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX) and insulin. As shown in Fig.3.7A, WT C/EBPα, I294A and R297K underwent adipocytic differentiation upon hormonal induction. However, differentiation was essentially impaired by R297A, R297Q, or BRM2, as judged by cellular morphology and cytoplasmic lipid accumulation. C/EBPα expression level was controlled by western blot (Fig.3.7B). Activation of the adipogenic gene aP2, adipsin and transcription factor PPARγ were restored by expression of WT C/EBPα but not by the citrullination mimicking R297Q mutant in C/EBPα deficient cells. On the contrary, R297K mutant strongly enhanced fat differentiation, as demonstrated by increased transcription of adipocyte specific genes (Fig.3.7C). To determine whether residue 297 dependent DNA-binding ability is required for cell cycle arrest, we examined colony formation in C/EBPα-/- MEF cells supplemented with C/EBPα mutants. Consistent with the E2F repression in luciferase assay, I294A and R297K inhibited cell growth more efficiently and similarly as WT C/EBPα, whereas BRM2, R297A, and R297Q display severely abrogated cell proliferation arrest (Fig. 3.8). Thus, although the latter mutants bind to E2F-DP more avidly (Fig. 3.4), efficient DNA binding capacity is a pre-requisite for C/EBPα mediated repression on cell growth. In accordance with adipogenesis, R297K but only little R297Q mutants were found associated with PPARγ and aP2 promoters by ChIP (Fig. 3.9A). These data indicate that intact DNA binding capacity mediated by C/EBPα R297 contributes to its association with target promoters and responsive genes activation. It was suggested that that several E2F target promoters such as DHFR, E2F1 and PCNA contain potential C/EBP binding sites in proximity to E2F binding sites (Sebastian et al, 2005; Wang & Timchenko, 2005). One wonders whether DNA binding ability of C/EBPα is also required for association on such E2F target genes. We then performed ChIP analysis and the results showed that R297Q binding to the DHFR promoter was significantly reduced compared to WT C/EBPα or R297K (Fig. 3.9B). Taken together, these data demonstrate that R297 is critical to mediate the association of C/EBPα with both C/EBP genes and E2F target genes.

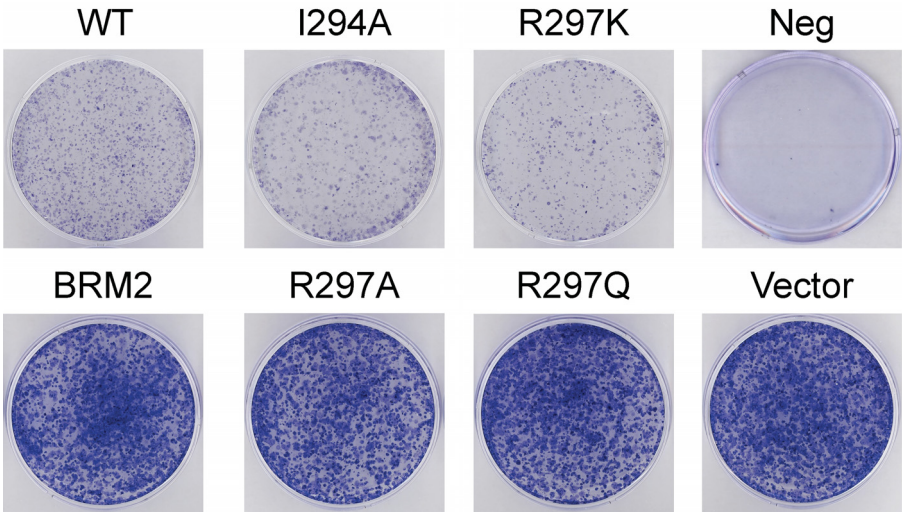

Figure 3.8: R297 is required for C/EBPα mediated cell growth control. C/EBPα-/- MEFs were stably transfected with empty vector or WT C/EBPα or mutants. After selection, 1×10^5 cells were seeded and stained with crystal violet after 6 days growth. Non-transfect cells served as negative control (Neg).

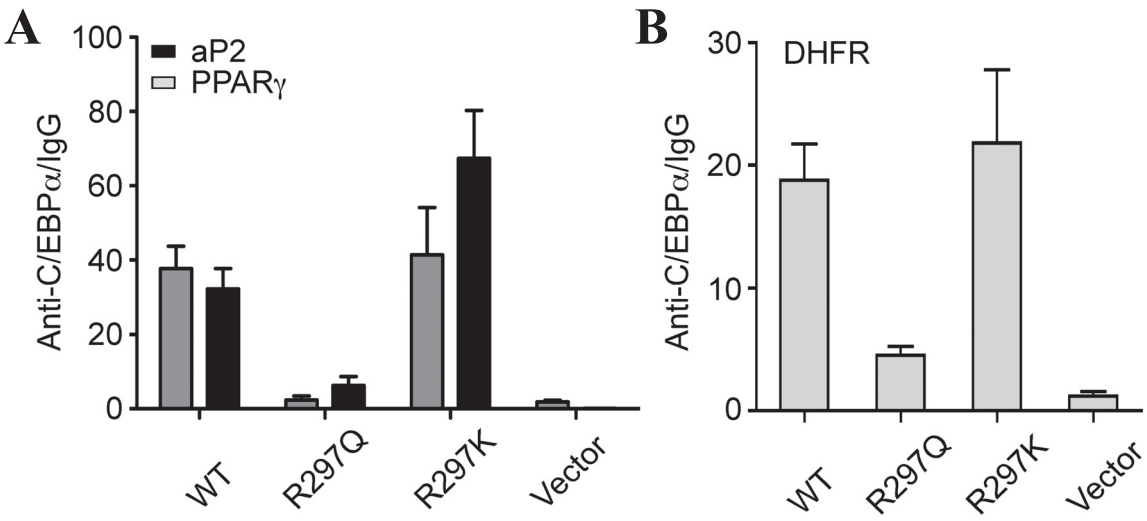

Figure 3.9: R297 mediates C/EBPα association to target promoters. A) ChIP analysis of C/EBPα-/- MEFs that were stably transfected with WT C/EBPα, R297Q, R297K or control vector in induced adipogenesis. Chromatin samples were immunopreciptated with antibody against C/EBPα or mouse IgG and analyzed by quantitative PCR, using primers flanking C/EBP-binding sites in the proximal promoters of the PPARγ2 and AP2 genes. ChIP quantification data are expressed as anti-C/EBPα versus IgG and normalized to input. B) Association of C/EBPα and mutants to E2F target DHFR promoter. All ChIP analysis were performed with cells harvest after 96 h treatment with adipogenic stimulation cocktail. Error bars indicate the means ±SD of three individual experiments.

3.3.6 PADI4 interacts with and citrullinates C/EBPα

Peptidylarginine deiminase 4 (PADI4) has a nuclear localization signal (NLS) and is mainly expressed in neutrophils granulocytes where C/EBPα plays a major role in differentiation (Hagiwara et al, 2002; Nakashima et al, 1999). It is also expressed in transformed cell lines, including HEK-293T cells (Chang et al, 2009) where we initially discovered C/EBPα citrullination. To examine whether PADI4 may interact with C/EBPα, HEK-293T cells were transfected with HA-PADI4 and C/EBPα and analyzed by immunoprecipitation. Subsequent immunoblotting showed that C/EBPα interacted with PADI4 (Fig. 3.10A). GST fusion proteins that are represented in Fig. 3.10C corresponding to functional domains of C/EBPα were purified form *E.Coli* extracts and tested for interaction with PADI4. As shown in Fig. 3.10B, PADI4 predominantly interacted with the C/EBPα basic region and to some extend with the leucine zipper domain. There is also some interaction with TEIII part. Next, *in vitro* citrullination was performed by incubating immunoprecipitated HA-tagged PADI4 from transfected cells with C/EBPα peptides. Immunoblotting, using an antibody recognizing chemically modified citrulline (α-MC) showed that the basic region and the middle part (TE-III) of C/EBPα could be citrullinated in the presence of Ca^{2+} by WT PADI4 but not by a catalytically inactive PADI4 mutant (HA-PADI4 D350A and D473A) (Fig. 3.11A). The functional consequence of the interaction between PADI4 and C/EBPα was examined by reporter assay on the C/EBP responsive cMGF promoter construct pM82. PADI4, but not the catalytic defective PADI4 mutant diminished transactivation in a dose dependent manner (Fig. 3.11B). In addition, the R297K mutant is largely resistant to PADI4 mediated repression. Altogether, these data suggest that PADI4 physically interacts and citrullinates C/EBPα, which lead to impaired DNA binding activity and diminished transactivation.

3.3.7 Knock down of PADI4 induces myeloid differentiation

We further examined the link between PADI4's inhibitory effects on C/EBPα activity and granulopoiesis in the myeloid precursor cell line 32D. The 32D cells may undergo C/EBPα dependent granulocytic differentiation in response to G-CSF. Interestingly, 32D cells express low level of both, C/EBPα and PADI4. Double-strand oligonucleotides was

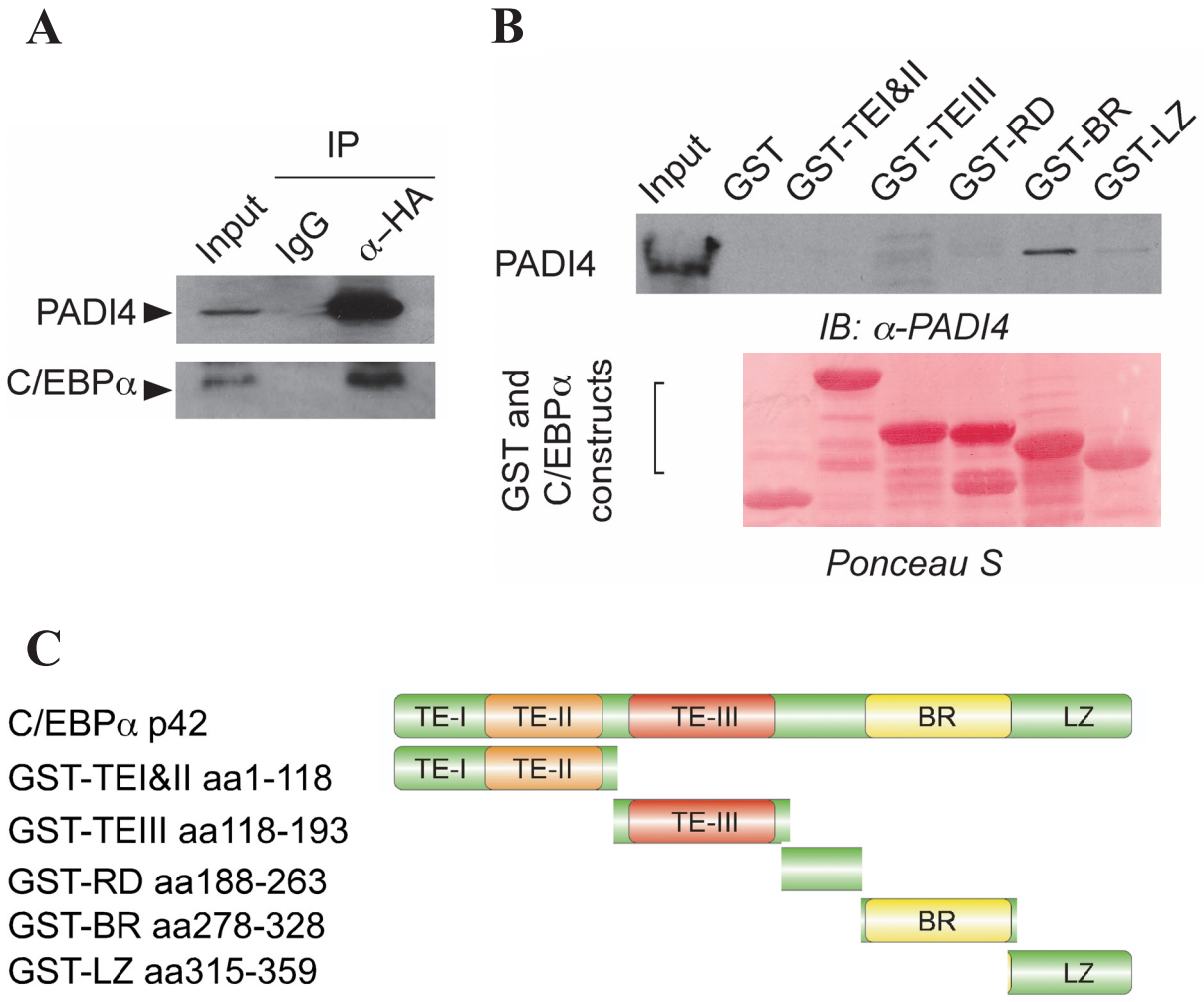

Figure 3.10: PADI4 interacts with C/EBPα basic region. A) Interaction of C/EBPα and PADI4. Lysates of HEK293T cells transfected with Flag-C/EBPα and HA-PADI4 expression vectors were immunoprecipitated with anti-HA antibody or mouse IgG, followed by immunoblotting with anti-C/EBPα. B) PADI4 interacts with the basic region of C/EBPα. GST-C/EBPα fragments immobilized on beads was incubated with lysates that transfected with HA-PADI4. GST protein served as negative control. Bound PADI4 was detected by immunoblotting with anti-PADI4 antibody and GST proteins were stained with Ponceau S. C) Schematic representation of GST fused C/EBPα fragments in GST pull down assay and following *in vitro* citrullination assays.

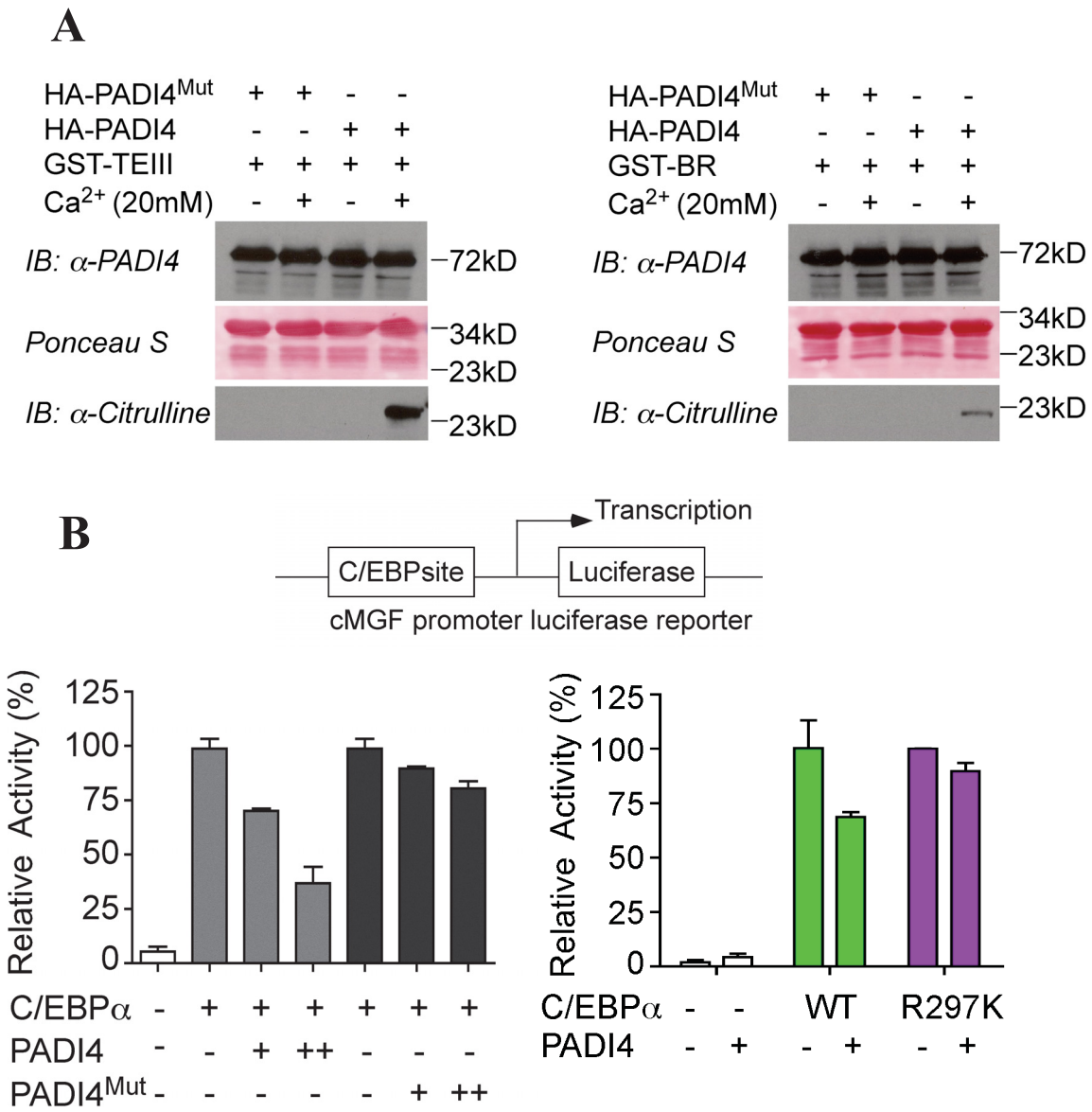

Figure 3.11: PADI4 citrullinates C/EBPα and represses C/EBPα mediated transcription. A) In vitro citrullination assay. HA-tagged PADI4 or mutated form were expressed in HEK-293T cells, purified from cell lysates by immunoprecipitation with anti-HA antibody and incubated with 4ug GST fused C/EBPα fragment in the presence of $CaCl_2$ at 37°C for 3 h, followed by immunoblotting with anti-PADI4 or anti-modified citrulline (α-MC) antibody. GST fused peptides were stained by Ponceau S. GST fused C/EBPα TE-III and basic region (BR) are shown in Figure 3.9. B) C/EBPα mediated transcription was inactivated by PADI4. HEK-293T cells were transfected with a C/EBP-responsive gene reporter (50ng), C/EBPα or R297 mutants (20ng) and wild-type PAD4 (150ng, 300ng) or catalytic inactive form of PADI4 (D350A and D473A) plasmids. Error bars indicate the means ±SD of three individual experiments.

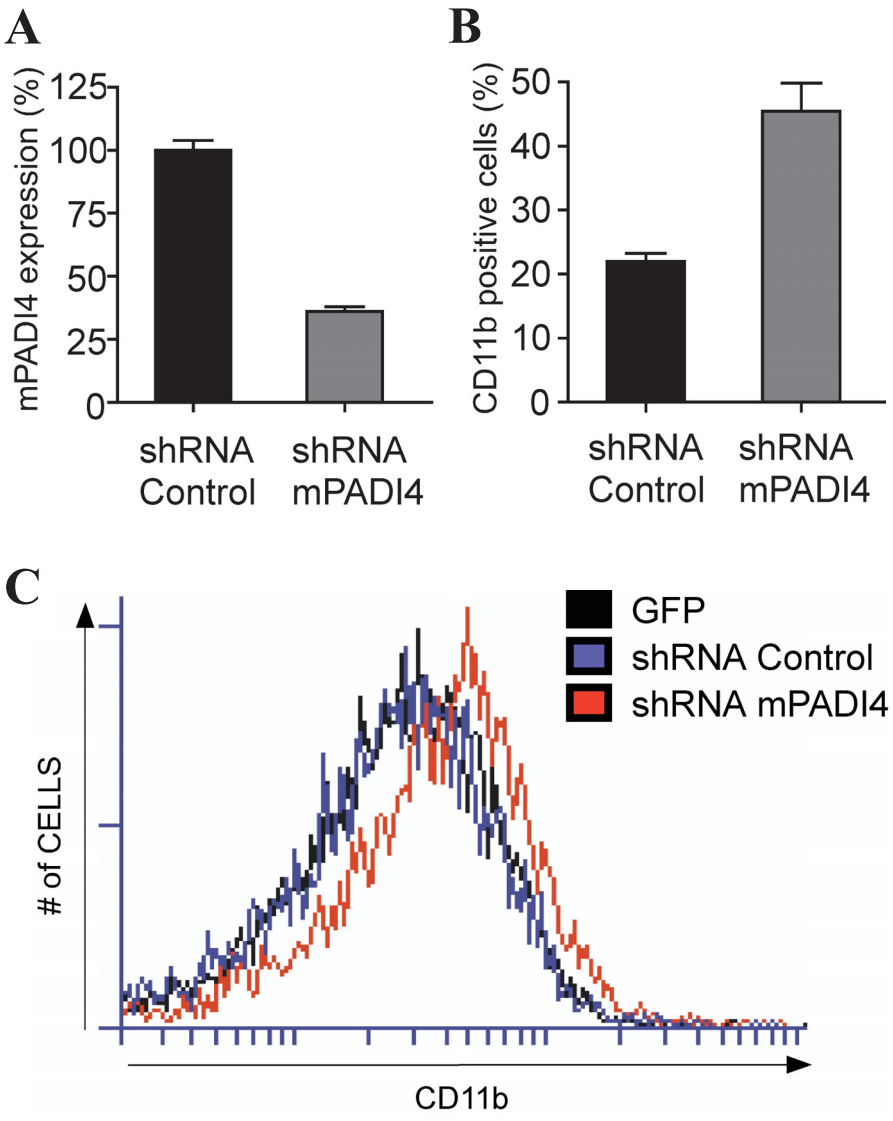

Figure 3.12: Knock down of PADI4 enhances myeloid 32D cell differentiation. A) shRNA (with IRES-EGFP) designed to knock down mouse (mPADI4) was electroporated into 32D cells and mPADI4 mRNA levels were analyzed by real-time RT-PCR 48 hr later. B) Percentage of CD11b positive 32D cells that are electroporated with shRNA control or shRNA to knock down mPADI4. Cells were gated for EGFP expression and assessed the CD11b myeloid marker with flow cytometry 48 hr after electroporation. Data are representative of three independent experiments. C) Histogram shows CD11b expression in 32D cells that are electroporated with shRNA control or shRNA to knock down mPADI4.

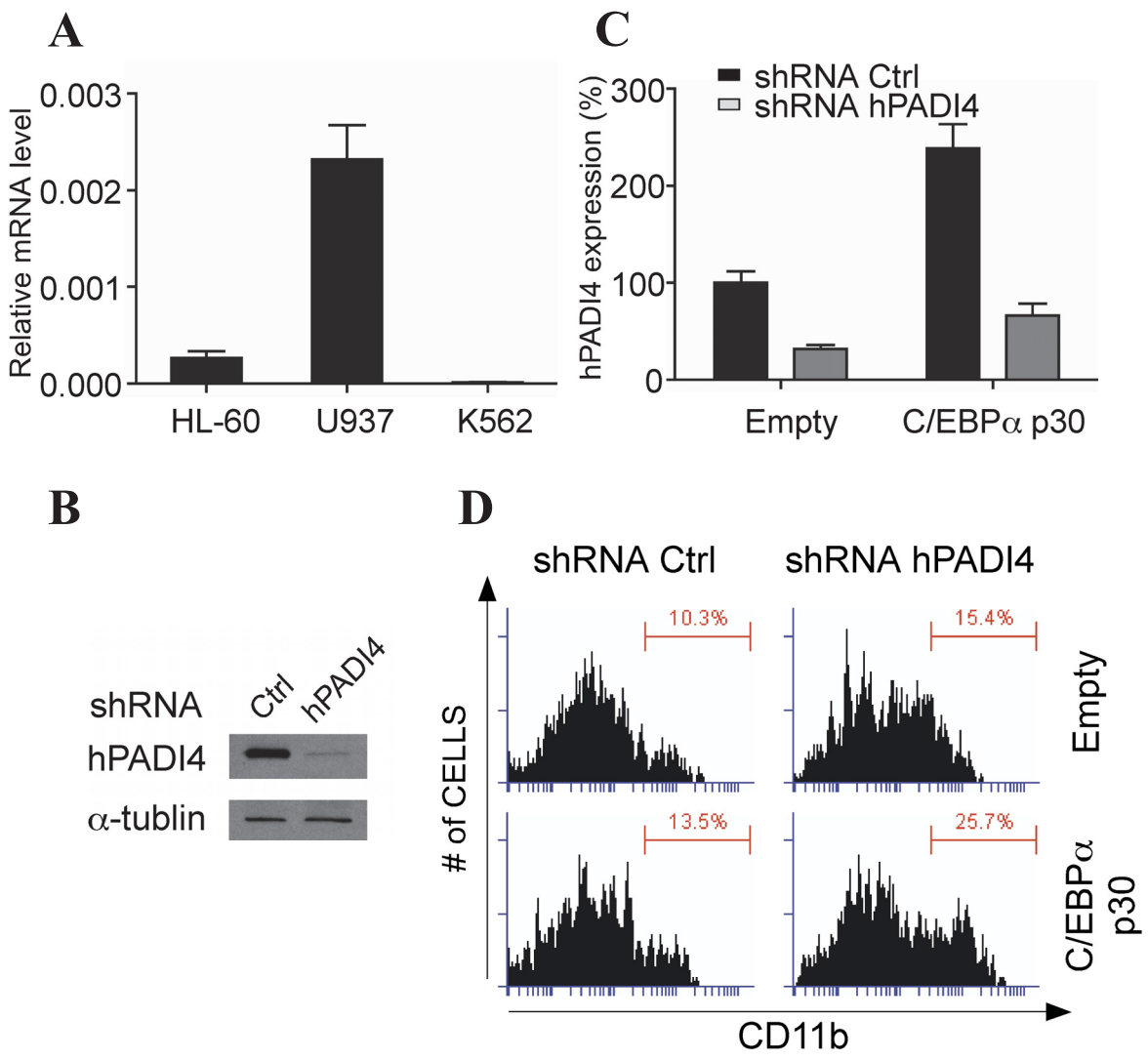

Figure 3.13: Knock down of PADI4 enhances human myeloid leukemia cell differentiation.

A) Expression of PADI4 in human leukemia cell lines. PADI4 mRNA levels in HL-60, U937 and K562 are analyzed by real-time RT-PCR and normalized to GAPDH. B) shRNA to knock down hPADI4 was electroporated into U937 cells and PADI4 expression was analyzed by western blot after 48 hr. C) C/EBPα p30 induces PADI4 expression. Oncoprotein C/EBPα p30 or shRNA to knock down hPADI4 was electroporated into U937 cells and PADI4 mRNA level was analyzed by real-time RT-PCR 48 hr later. D) PADI4 knock down released dominant negative effect of C/EBPα p30 and enhanced myeloid differentiation. CD11b expression of U937 samples in C) was assessed by flow cytometry. Data are representative of three independent experiments.

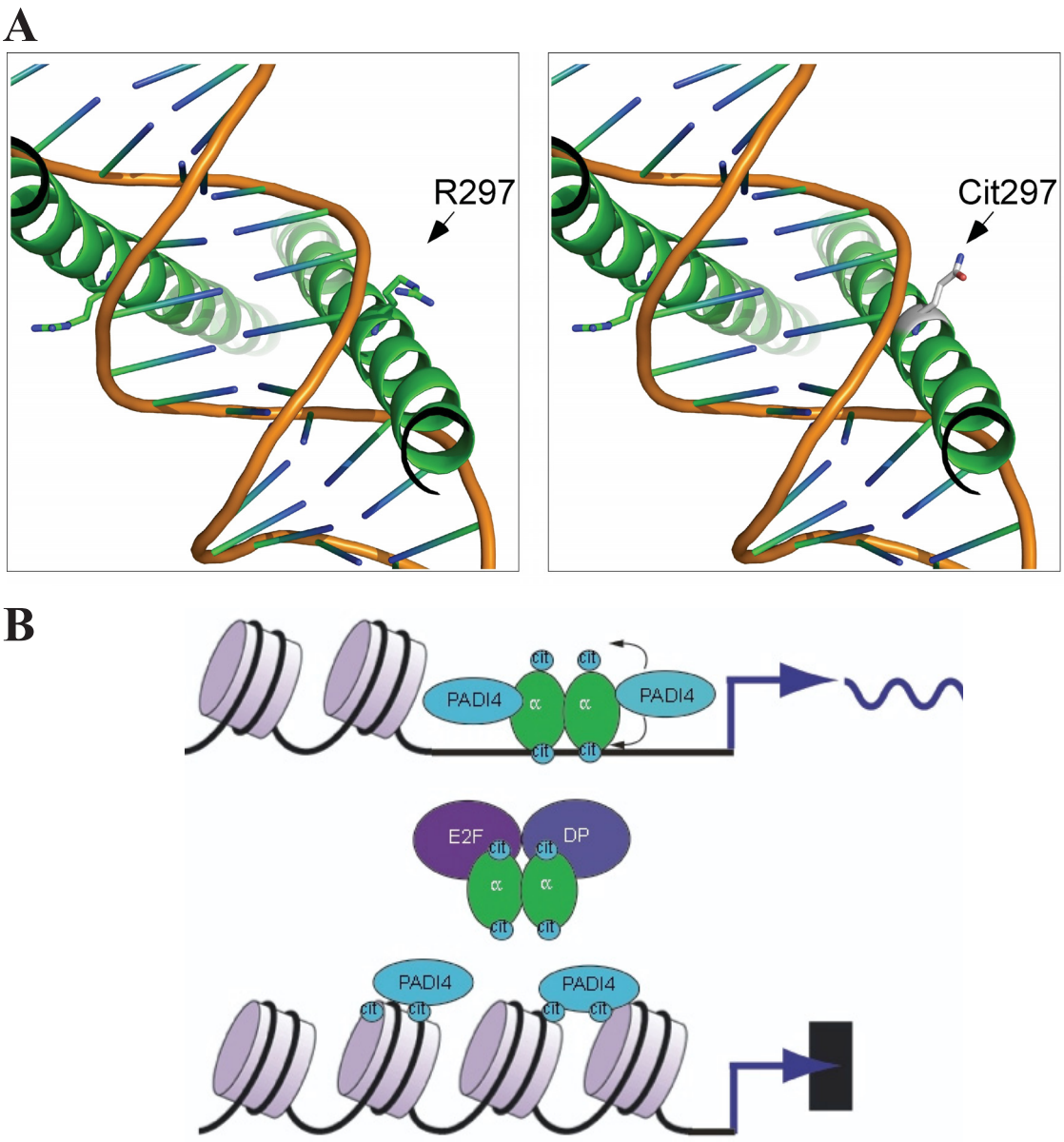

Figure 3.14: Model of PADI4 mediated citrullination on C/EBPα R297. A) Effects of citrullination on R297. Left: C/EBPα basic region α-helix is located in the major groove of DNA. Side chain of basic R297 (Arginine pKa=12.48) partially faces to DNA and mediates electrostatic interaction with negative charged DNA backbone; Right: citrullination removes positive charge and an imine from arginine. The neutral citruline has increased hydrophilicity and may face away from DNA to the solvent. B) PADI4 suppresses C/EBPα mediated transcription. Upper panel: C/EBPα activates the transcription of its target genes; Upon signaling, PADI4 binds to C/EBPα basic region and citrullinates arginines in TE-III and the basic region; Lower panel: citrullinated C/EBPα has diminished DNA binding affinity and increased binding to E2F/DP and is easily taken off from DNA binding sites. PADI4 may go on associating with chromatin and regulate gene transcription by citrullinating histone 3, histone 4 and certain other non-hisone proteins.

cloned into psiRNA-7SKGFPzeo vector (InvivoGen) and was applied to generate shRNA to knock down mouse PADI4 (mPADI4). We introduced vectors generating shRNA targeting PADI4 or off-target ShRNA into 32D cells by electroporation with Amaxa Cell Line Nucleofector device and reagent (details see methods). 48 hours post electroporation, the electroporated cells expressed GFP and were assayed for granulocytic differentiation by flow cytometry. As is shown in Fig. 3.12A, the shRNA construct reduced PADI4 mRNA level 48 hours after electroporation. Knock down of PADI4 induced elevated myeloid marker CD11b expression, even without G-CSF treatment (Fig. 3.12B). As is shown in Fig. 3.12C, FACS analysis demonstrated that the percentage of CD11b positive 32D cells increased with knocking down of PADI4.

It was observed that PADI4 is highly expressed in haematopoietic stem cells, oncoprotein transformed leukemia initiation cells (LICs) and many leukemia cell lines (Chang et al, 2009; Kirstetter et al, 2008; Krivtsov et al, 2006). This may indicate that PADI4 is involved in self-renewal. We compared PADI4 expression in human leukemic monocyte lymphoma U937 cell line, human promyelocytic leukemia HL-60 line and erythroleukemic K562 cells. As is shown in Fig. 3.13A, HL-60 cells and U937 cells expressed relatively high level of PADI4 as quantified by real-time PCR. We also observed that C/EBPα p30 induces increased PADI4 expression (Fig. 3.13B), which is consistent with previous reports that PADI4 is involved with leukemia initiation signature and can be induced by oncoproteins such as C/EBPα p30 and MLL-AF9 (Kirstetter et al, 2008; Krivtsov et al, 2006). We then designed shRNA targeting human PADI4 (hPADI4) and tested the efficiency in U937 cells (Fig. 3.13C). Since C/EBPα p30 induces PADI4, which inactivates C/EBPα by citrullination, we wonder whether the inhibition of PADI4 can overcome the increased PADI4 expression induced by C/EBPα p30 and restore the C/EBPα WT function. As is shown in Fig. 3.13D, enhanced myeloid differentiation by knocking down hPADI4 was observed in human leukemia U937 cells. We also found that p30 appeared to have some residual myeloid differentiation inducing activity. Moreover, block of PADI4 released the dominant negative effect of C/EBPα p30 and further promoted myeloid differentiation. It seems that the dominant negative effect of p30 is partially mediated by inducing PADI4 and in turn suppresses WT C/EBPα. Taken

together, our results suggest regulatory crosstalk between PADI4 and C/EBPα and that block of PADI4 can induce myeloid differentiation in both precursor cells and several leukemia cell line.

4 Discussion

4.1 Post translational modifications on C/EBPα

The data described in this thesis uncovered a set of signal dependent post translational modifications (PTMs) on C/EBPα. Such modifications may cause change in interaction partners, affecting gene regulation and epigenetic function of C/EBPα. In addition to reported PTMs, we detected novel mono-methylation, di-methylation and citrullination on several conserved arginines. On chromatin histones, arginine methylation and citrullination are tightly regulated by signals to antagonize each other and to alter the conformation of chromatin. One may expect that similar regulatory mechanism may also exist for certain C/EBPα functions. For example, the PTMs on R154 and R163 surrounding SUMoylation site in the regulatory domain might be involved in mediating SWI/SNF binding while PTMs on R323 and R343 may be involved in assisting or abrogating leucine zipper function. However, since such diverse modifications occur on so many sites, more work is are needed to define the contributuion of individual PTM and decipher the intricate network of protein interaction that are affected by these PTMs. Here, we concentrate on the modificiation of R297, an amino acid residue involved in the regulation of differentiation and proliferation control that has been found to occur as leukemia associated mutation.

4.2 PADI4 mediated citrullination on C/EBPα

We show here that, in support of a proposed "Indexing Code" notion (Kowenz-Leutz et al, 2010), PADI4 catalyzed citrullination and alters the function of C/EBPα. The impact of protein citrullination in cellular processes is an area that attracts increasing interest and contributes in understanding mechanisms of diseases and of pathology. Data presented here suggest that PADI4 mediated citrullination on C/EBPα basic region R297 diminishes C/EBPα -DNA binding affinity by converting the basic arginine to neutral citrulline. The positive charge of residue 297 is critical to stabilize protein-DNA complexes stability, and to adjust C/EBPα activity versus E2F-DP complex formation,

thus balance the reciprocal regulation of differentiation and proliferation. This mechanism provides an explanation as to the potential function of PADI4 in apoptosis and leukemia; and the switch between proliferation-differentiation in conjunction with E2F mediated gene regulation.

4.2.1 A role of PADI4 in suppressing C/EBPα function in stem cell and leukaemia

PADI4 has been recognized as an enzyme that delaminates C/EBPα R297. PADI4 is mainly expressed in granulocytes, monocytes and macrophage (Asaga et al, 2001; Nakashima et al, 1999). It is the only peptidylarginine deiminase that localized to the nucleus (Hagiwara et al, 2002). PADI4 is regarded as a key enzyme in demethylation mechanism to antagonize PRMT1/CARM1 function and reverse gene activation. Alternative mechanisms can be proposed such as dissociation of citrullinated histones from nucelosomes or the replacement with histone variants. Until now, histone H3, H4, p300, myelin protein and nucleophosmin (NPM1) have been identified as PADI4 substrates (Cuthbert et al, 2004; Lee et al, 2005; Tanikawa et al, 2009; Wang et al, 2004b; Wood et al, 2008).

PADI4 was originally identified in HL-60 cells upon differentiation to granulocytes, and was suggested to play a role in inflammation and the immune response. PADI4 mediated hypercitrullination decondenses chromatin as a prerequisite to form the neutrophil extracellular trap (NETs), which is an immune process to bacterial infection (Wang et al, 2009). On the other hand, accumulating reports supported the notion that PADI4 enhances apoptosis by affecting chromatin conformation. It was suggested that citrullination on basic arginines alters the net charge of histones and causes the nucleosomes to open up, which facilitates DNA fragmentation and histone degradation (Chang et al, 2005; Liu et al, 2006; Mastronardi et al, 2006). Interestingly, DMSO induced HL-60 differentiation and PADI4 expression are coupled with spontaneous apoptosis and coincides with decreased C/EBPα level (Santos-Beneit & Mollinedo, 2000; Scott et al, 1992). Besides, forced inactivation of C/EBPα or transfection of C/EBPα DNA binding-deficient mutants induces apoptosis in 32D myeloid precursor cells (Keeshan et al, 2006; Keeshan et al, 2003; Zhao et al, 2009), with the mechanism is not

clear. Probably PADI4 mediated citrullination and inactivation of C/EBPα contributes to change of chromatin structure and apoptosis progression in a context dependent manner. It was proposed that C/EBPα cooperate with NF-κB to induce anti-apoptotic Bcl-2 gene. Since C/EBPα core basic region (aa297-aa300) is responsible for the binding to NF-κB p50 (Paz-Priel et al, 2005), citrullination that occurs in this region may change the protein-protein interaction. In the peptide pull down assay, as shown in Fig. 3.4A, we observed increased binding of citrullinated C/EBPα peptide to NF-κB p50. However, it remains to be explored whether and how C/EBPα citrullination enhances apoptosis and whether this involves NF-κB.

Increased expression of PADI4 was monitored in patients with malignant tumors and a variety of tumor cell lines such as lymphoma Jurkat and leukemia U937. PADI4 expression was detectable in CD34+ stem cells and there are more CD34+ cells in PADI4 positive tumors than normal tissues, which suggest an association between precursor cell proliferation and PADI4 activities (Chang & Fang, 2010; Chang et al, 2009). Of particular attention is the finding that in the acute myeloid leukemia mouse model expressing C/EBPα dominant-negative mutant p30, PADI4 was significantly increased in granulocyte-macrophage progenitor (GMP) and committed myeloid leukemia-initiating cells (LICs) with an HSC-associated self-renewal signature, which was also observed in MLL-AF9 transformed leukaemia stem cells (Kirstetter et al, 2008). These observations suggest a role of PADI4 in leukemia initiation. In agreement with this report, we also observed increased expression of PADI4 by introducing C/EBPα p30 in 32D myeloid myeloid precursor cells (Fig. 3.13C). Recent studies showed that PADI4 associates with HDAC and directly inhibits p53 target gene expression such as p21. Transient activation of p53 increases histone arginine methylation and a decrease of citrullination on the p21 promoter. Also, an increase of PAD4 was detected at the p21 promoter and coincides with loss of RNA Pol II (Li et al, 2008). Morevoer, citrullination of Inhibitor of Growth 4 (ING4) by PADI4 increased susceptibility of ING4 to degradation and disrupts interaction between ING4 and p53, which further attenuates p53 activity (Guo & Fast, 2011). In a genome-wide analysis to investigate PADI4 target genes in MCF-7 breast cancer cells, ChIP on chip data revealed that PADI4 facilitates Elk-1 phosphorylation as a

co-activator in c-Fos expression (Zhang et al, 2011). Interestingly, PADI4 association targets are positively correlated with the ChIP-chip data of gene activation including E2F1 and PolI mediated transcription (Zhang et al, 2011). The activation of E2F by PADI4 may favor cell cycle entry and may account for leukemia-associated features. Moreover, large amount of the active genes associated with PADI4 binding are involved in RNA processing and ribosomal biogenesis (Zhang et al, 2011). Taken together, PADI4 has multiple functions in tumorgenesis such as suppressing tumor suppressor genes and actively transcribed genes related to cell cycle and growth.

C/EBPα activity is indispensable in myeloipoiesis and abrogation of C/EBPα has often been reported in leukemia. AML-ETO and BCR-ABL translocation products mediate suppression on C/EBPα on the transcriptional and translational level (Pabst et al, 2001a; Perrotti et al, 2002). Tribbles homolog 1 and 2 inactivate C/EBPα by degradation and induces acute myelogenous leukemia (Keeshan et al, 2006). Ectopic expression of C/EBPα overcomes the inhibitory factors and induces differentiation of such leukemic cells. Numerous mutations on CEBPA genes were identified from around 9% AML patients (Nerlov, 2004), which can be divided into two types: N-terminal mutations that lead to increased translation of truncated p30 isoform and C-terminal mutations that result in defect DNA-binding or dimerization. The p30 isoform does not possess a major part of the transactivation domain and is defect for E2F repression (Porse et al, 2001). Surprisingly, although the bZIP domain is kept intact, p30 shows reduced DNA binding and exert a dominant-negative effect on wild-type C/EBPα (Cleaves et al, 2004; D'Alo et al, 2003; Pabst et al, 2001b). In this work we found that in myeloid precursor 32D cells transfected with C/EBPα p30 and leukemic cell line such as HL-60 or U937, PADI4 expression is unregulated. Our date show that PADI4 mediates citrullination on C/EBPα and suppresses transactivation by C/EBPα. The citrullination on basic region affects DNA binding affinity, thus we propose that expression of p30 isoform decreases C/EBPα DNA binding and blocks p42 function partially by the induction of PADI4 and citrullination. Accordingly, U937 cells that had been blocked in PADI4 expression underwent myeloid differentiation. Moreover, in myeloid precursor 32D cells, knock down of PADI4 alone can enhance expression of the myeloid differentiation marker

CD11b. The potential connection between PADI4 suppression on C/EBPα and self-renewal or proliferation disorders needs further investigations. Taken together, our results suggest PADI4 as a part of oncogenic machinery that promotes leukemia through a mechanism involving inactivation of C/EBPα.

4.2.2 C/EBPα and E2F-DP complex interplay

The interplay between C/EBPα and E2F is an important pathway that regulates proliferation versus differentiation. In addition to the ability to direct terminal differentiation, C/EBPα functions as a tumor suppressor by arresting cell cycle. Mouse models showed that the combination of truncated p30 isoform and C-terminal mutations accelerated the progress of leukemogenesis. The N-terminal mutation permits the formation of committed myeloid progenitors and C-terminal mutations induced proliferation disorder (Bereshchenko et al, 2009; Porse et al, 2005). It was already known that C/EBPα causes cell cycle arrest by repressing E2F target genes, which is also a prerequisite for inducing adipogenic and myeloid genes to drive terminal differentiation. A recent notion suggested that E2F favors the clonal expansion phase but eventually counteracts adipogenesis by interfering with C/EBPα activity. The regulatory axis through PADI4 may provide the basis to an improved understanding how C/EBPα and E2F-DP activities interplay to switch proliferation and differentiation.

The basic region mutant BRM2 served as a paradigm to study C/EBPα regulated differentiation and leukemic initiation. BRM2 fails to support differentiation and to repress E2F mediated transcription, which was explained by defective binding to E2F, abnormal binding to DP or impaired DNA binding ability (Miller et al, 2003; Porse et al, 2001; Zaragoza et al, 2010). It is clear that certain substitutions on critical residues in the basic region of C/EBPα may change the α-helical structure and disturbs the binding interface with DNA. For example, the R305P mutant identified from AML patients and BRM5 Y285A were found to be dominant-negative forms with defective DNA binding (Asou et al, 2003). Although the contribution of R297 to DNA binding was proposed by structural analysis and R297P substitution was identified in an AML M2 subtype patient (Benthaus et al, 2008; Miller et al, 2003), however, no distinguishable changes of DNA

binding mediated by R297 mutants have been clearly demonstrated in previous research. Data presented in Fig. 3.3 indicate that in the BRM2 mutant the defect of DNA binding affinity to R297A is compensated by an increase of DNA binding caused by I294A, suggesting a complex phenotype of the double point mutant BRM2. In reporter studies BRM2 and R297 mutants achieved comparable activated transcription with WT (Keeshan et al, 2003; Zaragoza et al, 2010). Nevertheless, E2F-DP inhibits transactivation by R297A more efficiently than of WT C/EBPα. The differentiation activity of C/EBPα BRM2 can be rescued by siRNA that diminish endogenous E2F-DP (Zaragoza et al, 2010). Accordingly, E2F-DP activity dominates C/EBPα BRM2 mutant on both E2F target S-phase genes and on C/EBPα differentiation genes.

The data on citrullination of C/EBPα R297 provides a new insight for the critical regulatory role of bZIP domain of C/EBPα. PADI4 deiminates arginine and results in the neutral citrulline (side chain similar to Asn or Gln), which alters the protein charge. The crystal structure of C/EBPα basic region suggested that R297 interacts with DNA backbone (Miller et al, 2003). In agreement with this report, ITC measurement results demonstrated that BRM2, R297A and R297Q have diminished DNA binding affinity, whereas I294A and R297K showed increased binding to DNA (Table 3.1). EMSA data suggested that the DNA binding specificity was not changed, but the DNA-complex stability was reduced (Fig. 3.3). The positively charged arginine side chain (pKa=12.48) is critical to keep intact electrostatic interaction with the negatively charged DNA, which is required to stabilize C/EBPα on its binding site. Our previous work showed that E2F/DP complex inhibited C/EBPα by interfering with its binding to DNA (Zaragoza et al, 2010). Here we show that when neutral Ala or Gln substitutes R297, the mutant proteins are easily repressed by E2F/DP complex, which was also observed with the BRM2 mutants. After mutating R297 back to Lys to regain the positive charge, C/EBPα is stabilized on DNA target and is partially protected from E2F/DP repression. In accordance with this, WT C/EBPα and R297K but not R297Q, efficiently bound to a C/EBPα responsive gene promoter and induced adipocytic gene expression.

Several studies have proposed that C/EBPα represses E2F/DP regulated genes by

interacting with E2F protein at cis-regulatory E2F consensus sites on DNA (Porse et al, 2001; Slomiany et al, 2000). Although BRM2 failed to physically interact with E2F4, binding affinity to E2F1 remained unchanged (D'Alo et al, 2003; Keeshan et al, 2003). In agreement with this, all our mutants bind to E2F1 as well as to DP1. However, data presented here suggest that unimpaired DNA binding ability of C/EBPα is a prerequisite for the repression on E2F and the resistance to E2F-DP inhibition (Fig. 3.3 and Table 3.1). Our data support the notion that repression of E2F target genes requires not only the interaction between E2F-C/EBPα, but also the DNA binding ability of C/EBPα. Previously, it was reported that several E2F target promoters such as E2F1, DHFR and PCNA contain C/EBP binding site adjacent to E2F binding site (Sebastian et al, 2005; Wang & Timchenko, 2005). Consistently, it was shown that C/EBPα BRM2 failed to bind to DHFR probe and C/EBPβ S273A with compromised DNA binding displayed diminished association with DHFR and PCNA promoter (Lee et al, 2010; Porse et al, 2001). Although C/EBPβ S273 is not at equivalent position as R297 in C/EBPα, mutation on this residue impairs dimerization and DNA binding ability of C/EBPβ. Therefore, it is likely that C/EBPα DNA binding ability is indispensable and a triple E2F-DNA-C/EBPα interaction is required for effective repression on certain E2F targets.

4.2.3 Model and future plans

According to the experimental data shown here, the following model is suggested: PADI4 converts the positive C/EBPα R297 to none charged citrulline and destabilizes DNA-protein complex formation, which results in C/EBPα dissociation from its target genes for differentiation. The inability of C/EBPα to bind to E2F-sites abrogates repression of E2F mediated S-phase genes transcription and cell cycle progress. Citrullination of C/EBPα R297 decreases DNA binding while increases E2F-DP interaction, thus affecting the equilibrium of C/EBPα-E2F activities and regulating differentiation–proliferation switch.

Inhibition and mutations of C/EBPα are often observed in AML. The disruption of C/EBPα DNA-binding is accomplished by mutations in the basic region DNA-binding domain and amino-terminal or carboxy-terminal deletion. Our data presented here

provide a mechanism for the regulation of WT C/EBPα and an explanation of the basic region mutation found in leukemia. C/EBPα harboring R297 mutant with impaired DNA binding affinity fails to induce adipogenesis or inhibit cell growth. According to our model, citrullination on R297 converts C/EBPα to be similar to the myeloproliferation inducing BRM2 mutant or R297Q mutant, which are constitutively repressed by E2F-DP complex. Therefore, over expression of PADI4 may cause C/EBPα dissociation form DNA and may determine malignancy in leukemia. It would be of interest to analyze whether PADI4 mediates disruption of C/EBPα's DNA-binding in AML patients. One would therefore expect that AML without C/EBPα mutation may have PADI4 up regulated. It is reasonable that therapies directed against PADI4 could reverse the malignant phenotype by inhibiting abnormal self-renewal and restore granulocytic maturation.

It was suggested that C/EBPα cellular localization is regulated by a nuclear localization signaling domain embedded in the basic region or togather with the N-terminal extended part (Muller et al 2010; Yin et al, 1996). In particular, the extended form of C/EBPα with phosphorylation on serine 299 mainly localizes in nucleolus, which stimulates rDNA transcription and cell growth (Muller et al 2010). As discussed above, genome wide analysis showed that large amount of the active genes associated with PADI4 binding are involved in RNA processing, RNA PolI activation and ribosomal biogenesis (Zhang et al, 2011). In addition, PADI4 catalyzed citrullination on NPM1 protein regulate its nucleolus localization and may subsequently affect shuttling of other proteins between the nucleolus, nucleoplasm and cytoplasm (Tanikawa et al, 2009). Taken together, one would ask whether citrulination of arginine 297 in extended-isoform C/EBPα regulates its nucleolus localization, witch is probably one pathway of PADI4 to promote ribosomal biogenesis via modulating extended form C/EBPα.

PADI4 may function as a transcriptional activator or repressor on different genes loci. Future analysis will address which of the C/EBPα target genes are repressed by PADI4 and which of the genes that have been shown to be activated by PADI4 are controlled by C/EBPα. Furthermore, it seems that PADI4 association on chromatin does not necessarily

only lead to histone citrullination, but may alter gene expression by catalyzing citrulliantion on non-histones. It would be interesting to illustrate the relationship among PADI4 association, histone citrullination and gene activation by system biology approaches. It may further help to find more PADI4 regulated pathways to ultimately understand PADI4 function in physiology and physiopathology.

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6 Abbreviations

µg microgram

µl microliter

µM micromolar

aa amino acids

APS Ammoniumpersulfate

AML Acute Myeloid Leukemia

BRM Basic Region Mutant

BSA Bovine serum albumin bp base pair(s)

bZIP basic leucine zipper C/EBP

CCAAT/Enhancer Binding Protein

CDK Cyclin-dependent kinase

Cit Citrullination

DHFR dihydrofolate reductase

DMEM Dulbecco's modified Eagle medium

DMSO Dimethylsulfoxid DNA Desoxyribonucleic acid

DP Dimerization Partner of E2F

DTT Dithiotreitol

E2F Early gene 2 factor

ECL Enhanced Chemiluminescence

E. coli Escherichia coli

EDTA Ethylenediaminetetraacetate

ER Estrogen receptor

FACS Fluorescence activated cell sorter

FCS Fetal calf serum

FITC Fluoresceinisothiocyanat

GFP Green-fluorescent protein

GST Glutathione S-transferase

HDAC Histone-Deacetylase

HEPES N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HRP Horseradish peroxidase
IBMX 3-isobutyl-1-methylxanthine IF Immunofluorescence
IPTG Iso-propylthio-β-D-galactopyranoside
IRES internal ribosome entry site
MEF Mouse Embryonic Fibroblast mg milligram
ml milliliter
mM millimolar
min minutes
mRNA Messenger-Ribonucleic acid ng nanogram nm nanometer
NP-40 Nonidet P-40
Oil Red O 1-8-[4-(Dimethylphenylazo)dimethylphenylazo]-2-naphthalenol
PADI Peptidylarginine deiminase
PAGE Polyacrylamide Gel Electrophoresis
PBS Phosphate Buffered Saline
PCR Polymerase-chain-reaction
PPARγ peroxisome proliferator-activated receptor γ
pRB Retinoblastoma protein
PRMT Protein arginine methyltransferases
PTM Post-translational modifications
PVDF Poly vinylidene difluoride
rpm Rotations per minute
RNA Ribonucleic acid
rRNA ribosomal RNA
siRNA small interference RNA
TEMED (N,N,N',N'-Tetramethylethylenediamine)
Tris Tris(hydroxymethyl)aminomethane
Triton X-100 Octylphenoldecaethylenglycolether
Tween-20 Polyoxyethylensorbitanmonolaurat
X-Gal 5-bromo-4-chloro-3-indolyl β-D-galactoside
WT wild type

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